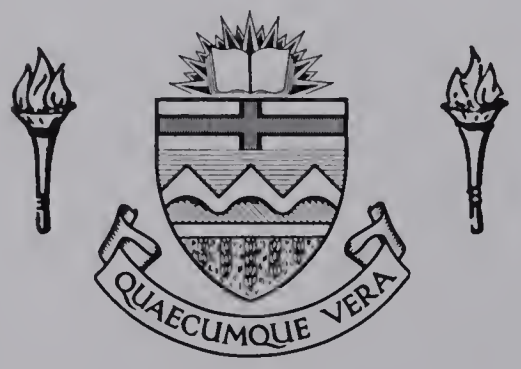

THE UNIVERSITY OF ALBERTA

THE CHANGES OF PSEUDOMONAS AERUGINOSA STRAIN MAC-264 AFTER PHAGE ϕ-2 INFECTION AND AFTER MITOMYCIN C TREATMENT

by

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The undersigned certify that they have read and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "THE CHANGES OF PSEUDOMONAS AERUGINOSA STRAIN MAC-264 AFTER PHAGE ϕ-2 INFECTION AND AFTER MITOMYCIN C TREATMENT", submitted by Charles T.C. Chow in partial fulfilment of the requirements for the degree of Master of Science in Microbiology.

ABSTRACT

This study consists of two parts, (1) the changes in Pseudomonas aeruginosa strain MAC-264 cells after phage ϕ-2 infection, and (2) the induction of a temperate phage by mitomycin C in MAC-264 cells.

Soon after ϕ-2 infection, the rate of DNA synthesis in MAC-264 cells was altered from that of the normal uninfected cells, while protein synthesis was unaffected until 60 minutes after infection. In contrast, the RNA synthesis in the infected cells stopped soon after infection. Ninety-five per cent of the cells lost their capability to form colonies 5 minutes after infection with a multiplicity of infection of 6 to 9. However, the optical density of the infected culture remained roughly constant for at least 120 minutes after infection.

The phage ϕ-2 one-step growth curve showed that it had an eclipse period of 30 minutes, latent period of 40 to 45 minutes, rise period 30 to 35 minutes, and a burst size of about 120 to 140 phage particles per infected cell with multiplicities of 0.01 to 150.

Phage synthesis was stopped when a suitable amount of chloramphenicol was added 10 minutes after infection, but progeny could still by synthesized if the same amount of chloramphenical was added 20 to 30 minutes after infection. However, the burst sizes were smaller in the latter cases. Fifty minutes after removal of chloramphenicol from the infected cultures, phage production resumed, indicating that the inhibitory effect of chloramphenicol was reversible.

The effect of mitomycin C on MAC-264 cells was studied with respect to cell viability, cell lysis, macromolecular synthesis and

the induction of a temperate phage, ϕ-MC. More than 99% of MAC-264 cells lost their colony-forming ability 30 minutes after addition of mitomycin C at concentrations of 2.5 μg/ml, or higher. The cellular turbidity continued to increase until 90 to 105 minutes after mitomycin C addition. A temperate phage, ϕ-MC, was induced from mitomycin C treated MAC-264 cells 60 to 75 minutes after exposure.

In mitomycin C treated cells, DNA synthesis stopped immediately. In contrast, both RNA and protein synthesis did not stop until 60 and 75 minutes respectively after addition.

The induced phage was found to be a short-tailed DNA phage with a hexagonal head about 55 mμ in diameter. This phage has a buoyant density of 1.468 g/ml in CsCl and its DNA-protein ratio is found to be 45:55. The particle weight is 1.68×10^{-16} g and the molecular weight 110×10^{6} daltons.

The mole G + C content in this temperate phage was found to be 45% while that of its host was 68%.

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LIST OF ABBREVIATIONS

DNA	-	deoxyribonucleate anion
RNA	-	ribonucleate anion
DNase	-	deoxyribonuclease
RNase	-	ribonuclease
M.I.	-	multiplicity of infection
I.C.	-	infective center(s)
CM	-	chloramphenicol
MC	-	mitomycin C
O.D.	-	optical density
EDTA	-	ethylenediamine tetra-acetate
PFU	-	plaque-forming unit(s)
r.p.m.	-	revolutions per minute
Tris	-	tris (hydroxymethyl) amino-methane

All temperatures expressed as degrees centigrade.

INTRODUCTION

Although bacteriophages for Pseudomonas aeruginosa have been reported since 1923 (Cancik, 1923; Combiesco et al., 1923; Pons, 1923), relatively few intensive studies have been made. Most of the studies done at that time and since then concerning Pseudomonas phages have been mainly on lysogeny and induction of temperate phages (Hadley, 1924; Rabinoweitz, 1934a, 1934b; Fastier, 1945; Warner, 1950; Holloway et al., 1960, 1962, 1964; Feary et al., 1963a, 1963b; Holloway, 1965). Until now, no complete studies on protein and nucleic acid synthesis of Pseudomonas phage have been reported. One reason is that most Pseudomonas strains are known to harbor a variety of latent particles ranging in complexity from temperate phages (Holloway et al., 1960) to pyocins (Kageyama and Eggami, 1962; Kageyama, 1964; Ishii et al., 1965). These latent particles make the system complex and the results difficult to interpret.

The T-even phages of E. coli have been well characterized (Adams, 1959; Sinsheimer, 1960; Stent, 1963). For these phages, the one-step growth curves, latent periods, rise periods, and burst sizes are approximately the same. In comparison Pseudomonas phages appear to vary considerably for some of these parameters. Feary et al. (1963a, 1964) found that phage 7v, isolated from a lysogenic strain of Pseudomonas aeruginosa, has a latent period of 20 minutes, a rise period of 10 minutes and a burst size of 150. Grogan and Johnson (1964) also reported some similar results for another Pseudomonas phage 2. In contrast, Boucher (1965) found a much longer latent period (60 minutes), rise period (60 minutes) and a smaller burst size (50) for Pseudomonas phages B and ϕ-16. These differences may

be due to the specific phage-host system involved.

Chloramphenicol has been known for a long time to inhibit the synthesis of protein without affecting that of DNA and RNA (Wisseman et al., 1964; Brock, 1961; Weisberger and Wolfe, 1964). Tomizawa and Sunakawa (1955) reported that the increase of DNA in T_2 -infected E. coli cells was completely suppressed by the addition of chloramphenicol up to 4 minutes following infection, but addition at later times showed progressively less inhibitory action depending upon the time. Addition of chloramphenicol after the 10th or12th minutes showed no appreciable effect on DNA synthesis.

Mitomycin C is an antibiotic isolated from Streptomyces caespitosus and has been shown to be a potent bactericidal, cytotoxic and mutagenic agent by Hata et al. (1956). Its bacteriocidal effect depends on the concentration and the time of exposure (Iyer and Szybalski, 1963) and the lethal effect is due to the changes in the structure of the bacterial DNA (Iyer and Szybalski, 1963, 1964). It selectively inhibits the synthesis of DNA, but RNA and protein formations are not affected under appropriate conditions (Shiba et al., 1958, 1959; Sekiguchi and Takagi, 1959, 1960). DNA synthesis can be restored in inhibited cells by infection with virulent bacteriophages. The restored synthesis resulted in the production of mature phage (Sekiguchi and Takagi, 1959, 1960).

The ability of mitomycin C to induce phage production in lysogenic bacteria has also been described and include studies in E. coli K 12 (λ) (Korn and Weissbach, 1962; Otsuji et al., 1959; Levine, 1961), in E. coli 15 (Endo et al., 1965), in Xanthomonas campestris (Sutton and Quadling, 1963), and in Bacillus subtilis (Seaman et al., 1964),

and in B. stearothermophilus (Welker and Campbell, 1965).

Mitomycin C is used in this study to examine the phage ϕ-2 synthesis inside the mitomycin C treated cells. In addition to the expected results, a temperate phage is induced in the control culture which is treated with mitomycin C, but not infected with phage ϕ-2.

That the buoyant density of DNA in CsCl is directly proportional to its guanine-cytosine (G + C content) was shown by Sueoka (1959), Marmur and Doty (1959), Rolfe and Meselson (1959) and Schildkraut et al., (1962). Many advantages in this method of determining base composition include the use of impure DNA extracts, the small quantity required and the short time needed for the determination. However, there are also certain distinct disadvantages, the most important ones being the presence of odd bases and of glucose which affect the buoyant density of the DNA (Schildkraut et al., 1962).

The purpose of this study is to investigate the changes in Pseudomonas aeruginosa strain MAC-264 cells after infection with a virulent phage ϕ-2, the induction of a temperate phage from MAC-264 cells by mitomycin C, and the properties of this induced phage.

MATERIALS AND METHODS

1. Bacterial Strains and their Maintenance

Pseudomonas aeruginosa strains MAC-264 and PAE-2-1 were obtained from laboratory stock culture. Origin of MAC-264 is from a culture collection at Macdonald College, P.Q., and PAE-2-1 is from a culture collection at National Research Council, Ottawa.

They were maintained as stock cultures on trypticase soy slants (Difco) at 4°. The stock cultures were transferred to fresh media every 4 weeks.

2. Growth Conditions of Bacteria

The bacteria were grown in nutrient broth (0.8% Difco nutrient broth in distilled water) in a 37° water-bath shaker.

Prior to each experiment, the cells were incubated by adding 0.2 ml of overnight culture to 100 ml warm nutrient broth in a 37° water-bath shaker with constant shaking for 2 to 3 hours to obtain a logarithmically growing culture.

3. Phage Strains

Phage ϕ-2 -- this was isolated from sewage by Dr. Yamamoto of this department.

Phage ϕ-MC -- this was the terminology given to a temperate phage induced in *P. aeruginosa* strain MAC-264 by mitomycin C treatment.

4. Preparation of High Titer Phage Stock

The soft-agar (0.7% agar in nutrient broth) layer method described by Hershey et al (1943) and outlined by Adams (1959, p. 457) was used. Two drops of an overnight bacterial culture were mixed in soft nutrient

agar with enough phage, usually 10^5 to 10^6 PFU, to give confluent lysis on the plates. The mixture was poured on a nutrient agar plate (1.5% agar in nutrient broth) and incubated overnight at 37°. The phage were harvested by the method described in Adams (1959, p. 456). Ten ml of nutrient broth were poured on the plate, and allowed to stand for 15 to 20 minutes. Then the soft-agar layer and nutrient broth were scraped into a beaker and mixed on a magnetic stirrer for 30 minutes to elute the phage from the agar. Agar and bacteria were removed from the mixture by centrifugation for 10 minutes at 7,000 r.p.m. in a SS 34 rotor of the Serval RC-2 centrifuge. The supernatant obtained was centrifuged for 30 minutes at 25,000 r.p.m. in a type 30 rotor of the Backman Model L-2 Ultracentrifuge. The pellets were resuspended to about one-tenth of the original volume in 0.04 M phosphate buffer, pH 7.0 ($Na_2HPO_4.7H_2O$, 5.35 g; KH_2PO_4, 2.73 g; Ion exchange water, 1 liter). This was the phage stock used for further analysis.

5. Bacterial Growth Curve

A flask containing 100.0 ml of nutrient broth was inoculated with an overnight culture of P. aeruginosa strain MAC-264 or PAE-2-1 cells, and the optical density was adjusted to 0.05 at 600 mμ in a Bausch and Lomb Spectronic 20. The flask was incubated in a 37° water-bath shaker with constant shaking, and optical density measured every 15 minutes. The number of viable cells was determined by serially diluting the culture and mixing 0.1 ml of the desired dilutions with 2 ml soft nutrient agar and plated on nutrient agar plates, in duplicate, and incubated overnight at 37°. The colonies were counted on a New Brunswick Scientific Counter.

6. Determination of Rate of Adsorption of Phage ϕ-2 to MAC-264 Cells

This was determined by the method described in Adams (1959, p. 466).

Two-tenth ml phage ϕ-2 was added to 1.8 ml exponentially growing MAC-264 cells to give a multipilicity of infection ranging from 6 to 9. The mixture was incubated in a 37° water-bath shaker with constant shaking for 5 minutes. One-tenth ml aliquots were removed and diluted to a desired dilution in warm nutrient broth, and both infective centers and viable cells were determined by soft-agar layer method and plated on nutrient agar plates.

7. Addition of Phage ϕ-2 Antiserum

A phage ϕ-2 antiserum (1948), prepared by injecting a rabbit, was obtained from Dr. Yamamoto. Its velocity constant, K, was determined by the method described in Adams (1959, p. 463).

Two-tenth ml of phage ϕ-2 was added to 1.8 ml of 1/2000 antiserum diluted in warm nutrient broth and incubated for 5 minutes at 37°. Then, 0.1 ml of the phage-antiserum mixture was diluted in warm nutrient broth to give a final antiserum concentration which did not have any more phage inactivating action. The phage survival was determined by the soft-agar layer technique.

In order to inactivate 95 to 99% of the free phage from the phage-bacteria mixture, 0.2 ml of it was added to 1.8 ml 1/2000 antiserum dilution and incubated for 5 minutes. Then both infective centers and viable cells were determined.

8. One-Step Growth Curve of Phage ϕ-2

Two-tenth ml of phage ϕ-2 was added to 1.8 ml of exponentially

growing MAC-264 cells in nutrient broth at multiplicities of infection ranging from 0.01 to 150. After 5 minutes adsorption and 5 minutes antiserum treatment, the mixture was diluted in warm medium and incubated in a 37° water-bath shaker. Infective centers were determined by removing 0.1 ml aliquots every 5 minutes and using the soft-agar layer technique.

For intracellular phage growth, 2 ml aliquots were removed and mixed with 2 drops of chloroform. The mixture was shaken for 30 minutes, and assayed for infective centers.

9. Treatment with Chloramphenicol

Chloramphenicol was obtained from Parke-Davis & Co., Detroit, Mich., and dissolved in distilled water to give a final concentration of 2 mg/ml and stored at 4°.

One-tenth ml of chloramphenicol at a suitable dilution was added to 10 ml of bacterial cultures or to 10 ml of phage-bacteria mixture to give a final desired concentration. To remove its effect a 10^{-6} dilution was made in warm medium.

10. Addition of Mitomycin C to MAC-264 Cells

Mitomycin C (Sigma Chemical Co., St. Louis, Mo.) was dissolved in distilled water at a concentration of 1 mg/ml and stored at 4° covered with tin foil to avoid light.

It was added to cells at desired concentration and the culture incubated at 37° with constant shaking. The cell lysates were collected after complete cell lysis had occurred, usually 180 to 240 minutes after addition of MC.

11. Processing of MAC-264 Cell Lysates After MC Treatment

The cell lysates collected 180 minutes after addition of MC were separated from bacterial debris by low speed centrifugation. The supernatant was then centrifuged at 25,000 r.p.m. for 30 minutes in a Spinco Model L-2 Ultracentrifuge, rotor SW 25, and the pellet resuspended in 0.04 M phosphate buffer and stored at 4° in the presence of chloroform as described by Adams (1959).

12. Purification of Phage Ø-MC

One ml of the phage containing suspension was added to the top of a CsCl (Metheson, Coleman & Bell, Norwood, Ohio) stepwise gradient (densities ranging from 1.25 to 1.50 g/ml) and centrifuged at 35,000 r.p.m. for 3 hours in a Spinco Model L-2 Ultracentrifuge, rotor SW 39. Five drops per fraction were collected by piercing the bottom of the centrifuge tube. Each fraction was determined for its buoyant density, infectivity and optical density at 260 mμ.

All fractions containing high titer of phage Ø-MC were pooled and stored at 4° in the presence of chloroform for further study.

13. DNA Determination in Infected and MC-Treated MAC-264 Cells

This is basically a combination of DNA extraction by Sekiguchi and Takagi (1960) and the diphenylamine reaction described by Burton (1956). Ten ml aliquots of the bacterial suspension were chilled and rapidly acidified by addition of 10 N perchloric acid to give a final concentration of 0.5 N. After standing for 30 minutes in cold, the insoluble residue was collected by centrifugation and then heated with 1.5 ml of 0.5 N perchloric acid at 90° for 15 minutes in order to extract the DNA. After cooling, the suspension was centrifuged

at 10,000 r.p.m. for 10 minutes. The residue was saved for protein determination and 1 ml of the supernatant was added to 2 ml of diphenylamine reagent (1 g diphenylamine in 100 ml glacial acetic acid containing 2.75 ml concentrated sulfuric acid), and heated at 100° for 10 minutes and cooled. Its optical density was determined at 600 mμ in a Beckman DB-G Grating Spectrophotometer.

A standard curve was plotted by using calf thymus DNA purchased from Calbichem (Los Angeles, Calif.).

14. Protein Determination in Infected and MC-Treated MAC-264 Cells

The residue obtained above was redissolved in 1 ml of 1 N NaOH, and half of it was used to determine the protein content by the Folin phenol reagent according to the procedure of Lowry et al. (1951).

A standard curve was plotted by using bovine serum albumin purchased from Pentex, Inc., Kankakee, Ill.

15. RNA Determination in Infected and MC-Treated MAC-264 Cells

The RNA was determined by the method described by Mejbaum (1939). One ml of cell suspension was added to 3 ml of cold 0.5 N perchloric acid and centrifuged at 7,000 r.p.m. for 10 minutes. The pellet was resuspended in a mixture of 1.5 ml 0.1% $FeCl_3$ in concentrated HCl and 0.15 ml of orcinol reagent (100 mg orcinol per ml 95% ethanol), and heated at 100° for 30 minutes, then cooled rapidly and its optical density read at 670 mμ.

16. Isolation of Bacterial and Phage DNA

For studies on DNA it is necessary to separate the DNA from the cell components and in the case of phage from the protein coat.

The most satisfactory method of preparing the phage DNA was found

to be the phenol extraction procedure of Mandell and Hershey (1960). DNA was isolated from phage purified on a CsCl gradient as follows: 5 ml of phage suspension with a titer of about 1 X 10^{13} per ml were mixed with an equal volume of phenol on a Fisher Mini-shaker for 10 minutes. The emulsion was separated into three layers by a 10 minute centrifugation at 7,000 r.p.m. The upper layer containing the nucleic acid was removed with a pipette, transferred to a test tube and washed 5 times with an equal volume of ether. Traces of ether were removed by bubbling air through the mixture. Finally a 0.5 volume of isopropanol was layered on the DNA solution. The two layers were mixed with a glass stirring rod and the DNA was wound on the rod. The DNA fibers were wahed in 70% ethanol and then redissolved in 0.0015 M Tris buffer, pH 7.1.

The DNA was isolated from bacteria by the procedure of Marmur (1961). Five hundred ml of an overnight broth culture of bacteria were centrifuged for 10 minutes at 15,000 r.p.m. in a SS 34 rotor of the Servall RC 2 centrifuge. The pellet of bacteria was washed in 100 ml of 0.15 M NaCl plus 0.1M ethylenediaminetetreacetate (EDTA), pH 8 and centrifuged again at 15,000 r.p.m. for 10 minutes. To lyse the cells the bacteria were suspended in 15 ml saline-EDTA, to which was added 1 ml 25% sodium lauryl sulfate ($NaC_{12}H_{26}SO_4$). The mixture was then placed in a 60^{o} water bath for 10 minutes. The lysate was cooled, perchlorate added to a final concentration of 1 M, and the mixture shaken for 10 minutes with an equal volume of phenol on a Fisher Mini-shaker. The emulsion was separated into three layers by centrifugation at 7,000 r.p.m. for 10 minutes. The nucleic acid was removed and the procedure repeated until no protein appeared at the interphase.

The nucleic acid was then treated with ether, etc., as described previously in the isolation of DNA from phage.

The fibers of DNA were redissolved in 0.15 M NaCl plus 0.015 M trisodium citrate, pH 7.0. RNase (Calbiochem, Los Angeles, Calif.) was added to a final concentration of 10 μg/ml and the solution was incubated for 30 minutes at 37°. The DNA was again precipitated with 0.5 volumes of isopropanol, washed in 80% ethanol, and dissolved in 0.015 M NaCl plus 0.0015 M trisodium citrate.

17. DNA Density Determination by CsCl Gradient Centrifugation

Cesium chloride was dissolved in 0.005 M Tris buffer plus 0.005 M EDTA, pH 8.1. A CsCl gradient was formed by layering 1 ml fractions of CsCl solution with decreasing densities (1.85 to 1.45 g/ml). One ml of the isolated DNA was put on top of the preformed gradient and centrifuged at 35,000 r.p.m. for 3 hours in a type 39 rotor head of the Beckman Model L-2 Ultracentrifuge. Five drops per fraction were collected by piercing the bottom of the centrifuge tube. Each fraction was determined for its buoyant density and optical density at 260 and 280 mμ in a Beckman DB-G grating Spectrophotometer.

18. Preparation for Electron Microscopy

The purified phage Ø-MC from CsCl gradient centrifugation was dialyzed against 0.04 M phosphate buffer to remove CsCl, and then mixed with an equal volume of 3% phosphotungstic acid solution adjusted to pH 7.0 (Brenner and Horne, 1959). A droplet of this mixture was placed on a carbon-coated Formvar grid, excess fluid removed with a filter paper, and air-dried. The grids were examined in a Philips 200 Electron Microscope.

Samples of MC-treated MAC-264 cells for embedding and ultra-thin sectioning were prepared in the following way (Ryter and Kellenberger, 1958). Cultures of 200 ml were grown in nutrient broth in a 37° water-bath shaker to a concentration of 1 to 2 X 10^8 cells per ml. At given times, 10 ml samples were taken, to which OsO_4 fixative was added to a final concentration of 0.1%. The sample was then immediately centrifuged at 7,000 r.p.m. for 10 minutes, the bacterial sediment resuspended in 1 ml of fixative (1% OsO_4 in acetate-veronal buffer at pH 6.0, containing 0.01 M Ca^{++} and o.1% Bacto-tryptone), and fixed for 16 hours at room temperature. After a second centrifugation, the pellet was resuspended in 0.03 ml of 1.5% Noble agar (Difco) at 45° and deposited as a drop on a cold glass slide. After cooling, it was cut into small blocks, which were then washed for 2 hours in a 1% solution of uranyl acetate in the acetate-veronal buffer previously used. Finally these were dehydrated in an ethanol-water series and embedded in epoxy resins in gelatin calsules. The final embedding mixture contained the following four components (Glauert and Glauert, 1958),

1. 10.0 ml Araldite M
2. 10.0 ml Hardener 964 B
3. 1.0 ml Dibutyl phthalate
4. 0.5 ml Accelerator 964 C.

This mixture sets in about 48 hours at 60° to yield a light gold block with sufficient hardness ready to cut.

The blocks were sectioned with a MT-2 Porter-Blum Ultra-microtome to a thickness of about 60 mμ. The sections were mounted on carbon-coated Formvar grids, stained with uranyl acetate and lead citrate, and examined in a Philips 200 Electron Microscope. Electron micrographs were taken at magnification of 10,000 to 20,000.

RESULTS

1. Rate of Adsorption of Phage ∅-2 to Pseudomonas aeruginosa Strain MAC-264 and Survival of Cells after Infection

a) Determination of Velocity Constant of Adsorption:

Phage ∅-2 was added to exponentially growing MAC-264 cells at a multiplicity of infection (M.I.) of 7.6. Ninety-two per cent of the phages added to the cells were adsorbed in 5 minutes and the rate of adsorption followed the kinetics of a first-order reaction. The velocity constant of adsorption, K, was calculated according to the formula given in Adams (1959, pp. 466).

$$K = 2.3/(B)t \times \log p_o/p$$

in which, p_o = phage assay at zero time

p = phage not adsorbed at time t minute

(B) = concentration of bacteria as number per ml

In this experiment,

$p_o = 29 \times 10^8$

$p = 22 \times 10^7$

$t = 5$

$(B) = 42 \times 10^7$

therefore,

$K = 1.5 \times 10^{-9}$ ml/minute.

The results are shown in Table I and Fig. 1.

b) Survival of MAC-264 Cells After ∅-2 Infection:

Most of the cells lost their colony forming ability soom after infection with ∅-2 at a M.I. of 6 to 9. Only about 3% of the cells retained viability 5 minutes after infection, this is also shown in Table I and Fig. 1. The killing effect of ∅-2 in the first 5 minutes follows the kinetics of a first-order reaction.

TABLE 1

Adsorption of Phage ∅-2 to Pseudomonas aeruginosa Strain MAC-264 and the Cell Survival After Adsorption and Anti-∅-2-serum Treatment

Experiment No.			1	2	3
Input ∅-2/ml			20×10^7	110×10^7	101×10^7
Input MAC-264/ml			0.86×10^7	1.7×10^7	1.2×10^7
Apparent M.I.			2.2	6	8
After Adsorption and Centrifugation	I.C.		0.79×10^7 (92% expected)	1.73×10^7 (101% expected)	1.05×10^7 (90% expected)
	V.C.	expected	1.04×10^6	0.38×10^5	0.39×10^5
		found	0.37×10^6 (35% expected)	0.46×10^5 (110% expected)	0.38×10^5 (99% expected)
	Actual M.I.		3.0	6	8
After Antiserum and Centrifugation	I.C.	expected	0.79×10^6	1.73×10^6	1.05×10^6
		found	0.32×10^6 (41% expected)	0.76×10^6 (44% expected)	5.20×10^6 (50% expected)

The percent survival of infected cells without antiserum treatment shows that within the experimental error, one obtains the expected number. Antiserum treatment lowers the I.C. count by 50 to 60% (see also Table 11).

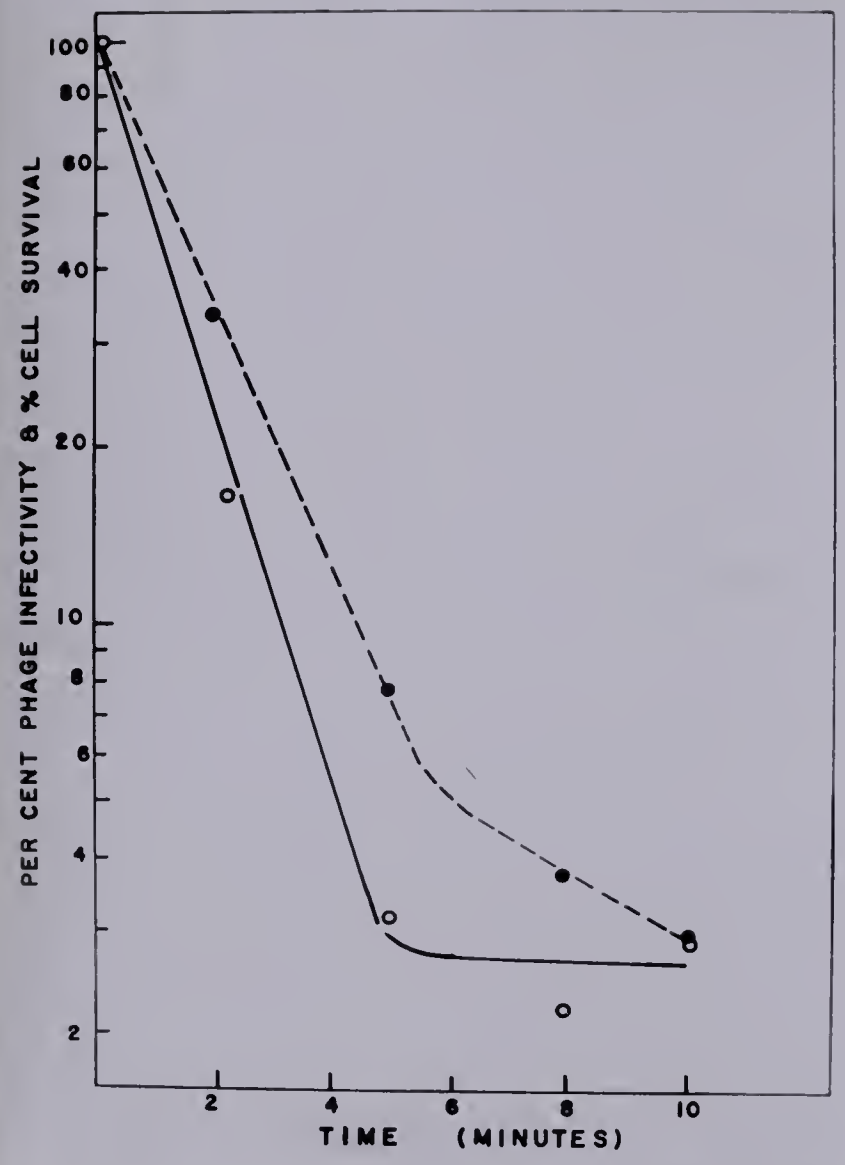
PER CENT PHAGE INFECTIVITY & % CELL SURVIVAL
100
80
60
40
20
10
8
6
4
2
2
4
6
8
10
TIME (MINUTES)

Figure 1. Rate of Adsorption of Phage ∅-2 to Pseudomonas aeruginosa Strain MAC-264 and Survival of Cells after Infection.

Two-tenth ml of phage ∅-2 was added to 1.8 ml exponentially growing MAC-264 cells at a multiplicity of infection of 7.6 at zero time. After adsorption in N.B. at 37° for different time intervals, a 1/100 dilution was made in warm N.B. and centrifuged at 7,000 r.p.m. for 5 minutes. The pellet was resuspended to the original volume and assayed for viable cell counts, and the supernatant was diluted and assayed for the remaining free phage particles.

0 _____ 0, Cell survival, pellet;

0 _____ 0, Free phage particles, supernatant.

2. Effect of Antiserum on Free Phage ∅-2, Uninfected and Infected MAC-264 Cells

a) Effect on Free Phage:

A phage ∅-2 preparation at a concentration of 3.71×10^9 PFU/ml was mixed with the antiserum and the final phage-serum dilution (D) was 2,000. The mixture was incubated for 5 minutes at 37°, and the phage not inactivated was found to be 22×10^6. The velocity constant, K, of this antiserum was calculated according to the formula (Adams, 1959, p. 463).

$$K = 2.3D/t \times \log p_o/p$$

$$= (2.3 \times 2000/5) \times \log 3.71 \times 10^9/22 \times 10^6$$

$$= 1940$$

b) Effect on Uninfected Cells:

No activation of normal MAC-264 cells by this serum was observed, and this is presented in Fig. 2.

c) Effect on Infected Cells:

When antiserum was added to infected cells for 5 minutes there is a loss of infective centers (I.C.). The results (Tables 1 and 11) show that the antiserum treatment eliminates 40 to 80 percent of infected cells. We do not know the reason for this killing effect. Nonetheless, comparison of intracellular phage growth curves from different experiments with different percent survival of infective centers (Figs. 7, 8 and 9) shows that only those infected cells that survive antiserum treatment are producing phage.

3. Effect of ∅-2 Infection on Turbidity of MAC-264 Cell Cultures:

Two tenth ml of concentrated phage ∅-2 were added to 100 ml exponentially growing *P. aeruginosa* strain MAC-264 cells at a density of

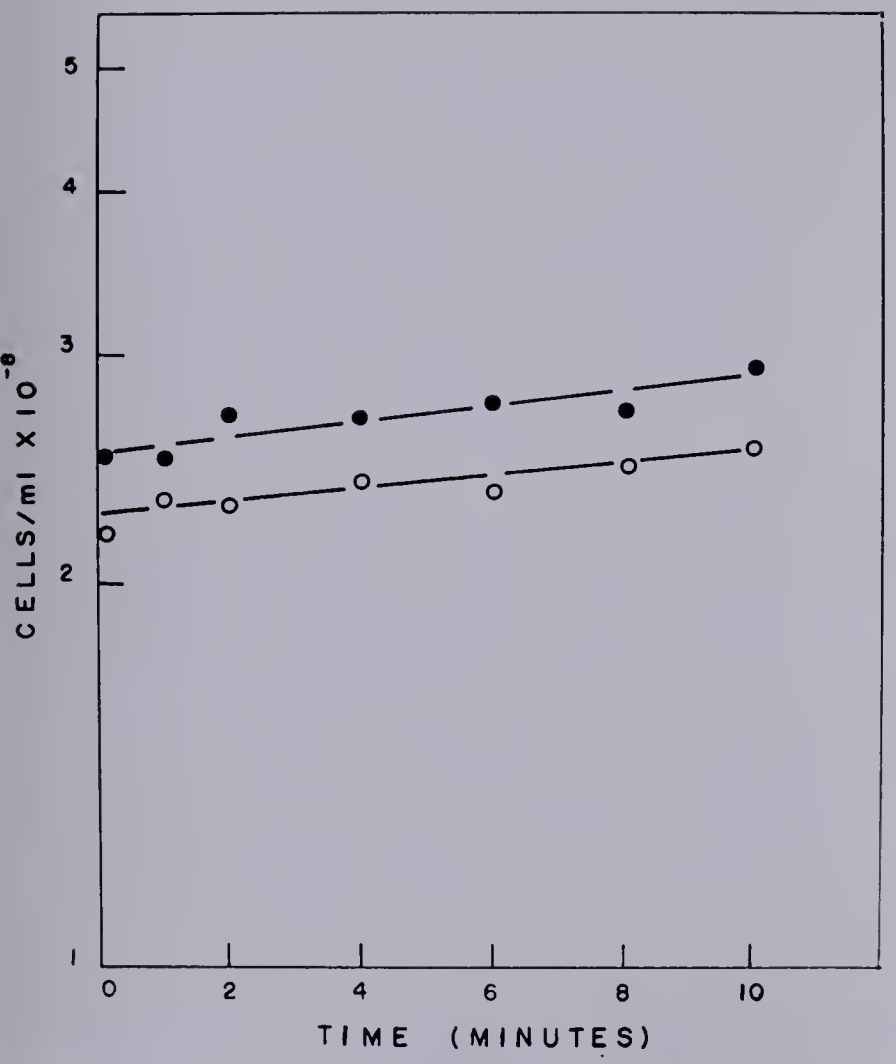
CELLS/ml X 10⁻⁸
5
4
3
2
1
0
2
4
6
8
10
TIME (MINUTES)

Figure 2. Effect of Phage Ø-2 Antiserum on P. aeruginosa strain MAC-264 Cells.

Two-tenth ml of antiserum was added to 1.8 ml exponentially growing MAC-264 cells to give a final serum dilution of 1/2000. Viable cell number was determined by plating 0.1 ml of the suspension on nutrient agar plates, in duplicate.

● ———— ●, Control, no antiserum;

0 ———— 0, Test, with antiserum.

TABLE II

Formation of Infective Centers of Pseudomonas aeruginosa Strain MAC-264 Cells After Phage Ø-2 Infection

Exp't No.	Cells per o.1 ml	M.I.	I.C./0.1 ml after serum treatment	I.C.-cells Ratio
1	9.4×10^6	3.5	1.8×10^6	20%
2	1.9×10^7	6.0	1.2×10^7	62%
3	6.2×10^6	8.0	2.7×10^6	44%
4	8.3×10^6	12.0	2.5×10^6	30%
5	2.0×10^7	15.5	6.8×10^6	34%
6	6.0×10^6	15.5	1.4×10^6	23%

1 - 2 X 10^8 cells/ml to give a multiplicity of infection of 7.5 and turbidities were measured at different times by the method described in Materials and Methods, and the results are shown in Fig. 3.

In the uninfected culture, the turbidity increased exponentially. On the contrary, the turbidity of the infected culture increased slowly for the first 20 minutes, and then remained at a constant level for as long as 120 minutes. This result indicates that in this phage-host system there is no visible lysis of the infected culture.

4. The Synthesis of DNA, RNA and Protein by Infected MAC-264 Cells

A bacterial culture at density of 1.2 X 10^8 cells/ml was divided into two portions. One was examined as an uninfected culture and the other as the infected one. Two-tenth ml of a Ø-2 concentrate was added to the infected culture to give a M.I. of 7.5. Aliquots were removed every 15 minutes during the first hour and every 20 minutes in the latter period of this experiment in order to determine the DNA, RNA and protein symthesis by the methods described in Materials and Methods.

a) DNA Synthesis:

In the uninfected culture, the amount of DNA doubles every 50 minutes. This increase was in agreement with the logarithmic increase in turbidity. In the infected culture, however, the rate of DNA synthesis was altered. Initially DNA synthesis rate was decreased for the first 10 minutes, but by the end of 20 minutes there was a marked increase in DNA beyond that found in the uninfected culture. This increase in DNA over the control culture was maintained until 50 minutes when the logarithmically increasing DNA in the uninfected culture began to surpass that of the infected cells. The total amount

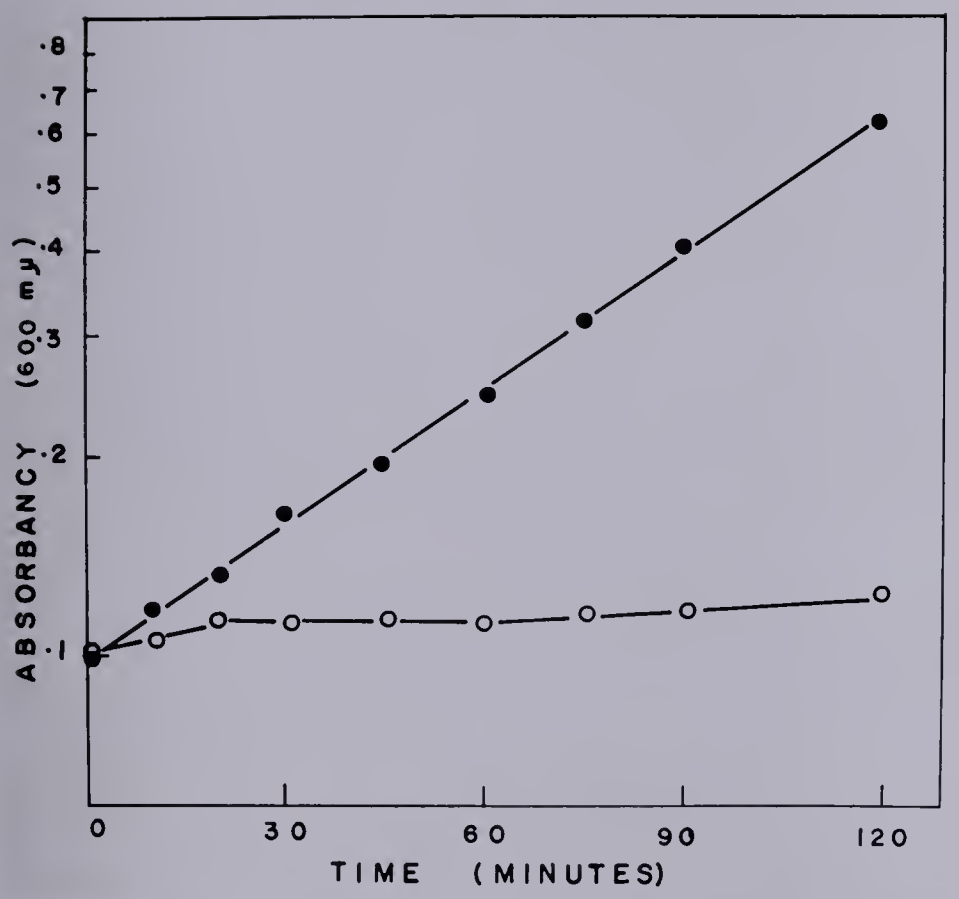
ABSORBANCY (600 mμ)
.8
.7
.6
.5
.4
.3
.2
.1
0
30
60
90
120
TIME (MINUTES)

Figure 3. Effect of Phage Ø-2 Infection on the Turbidity of P. aeruginosa MAC-264 culture.

Two-tenth ml of concentrated phage Ø-2 was added to 100 ml exponentially growing MAC-264 cells to give a multiplicity of infection of 7.5. The turbidities were measured at 600 mμ in a Bausch and Lomb Spectronic 20 at different time intervals.

● ———— ●, Control, Uninfected;

O ———— O, Test, Infected.

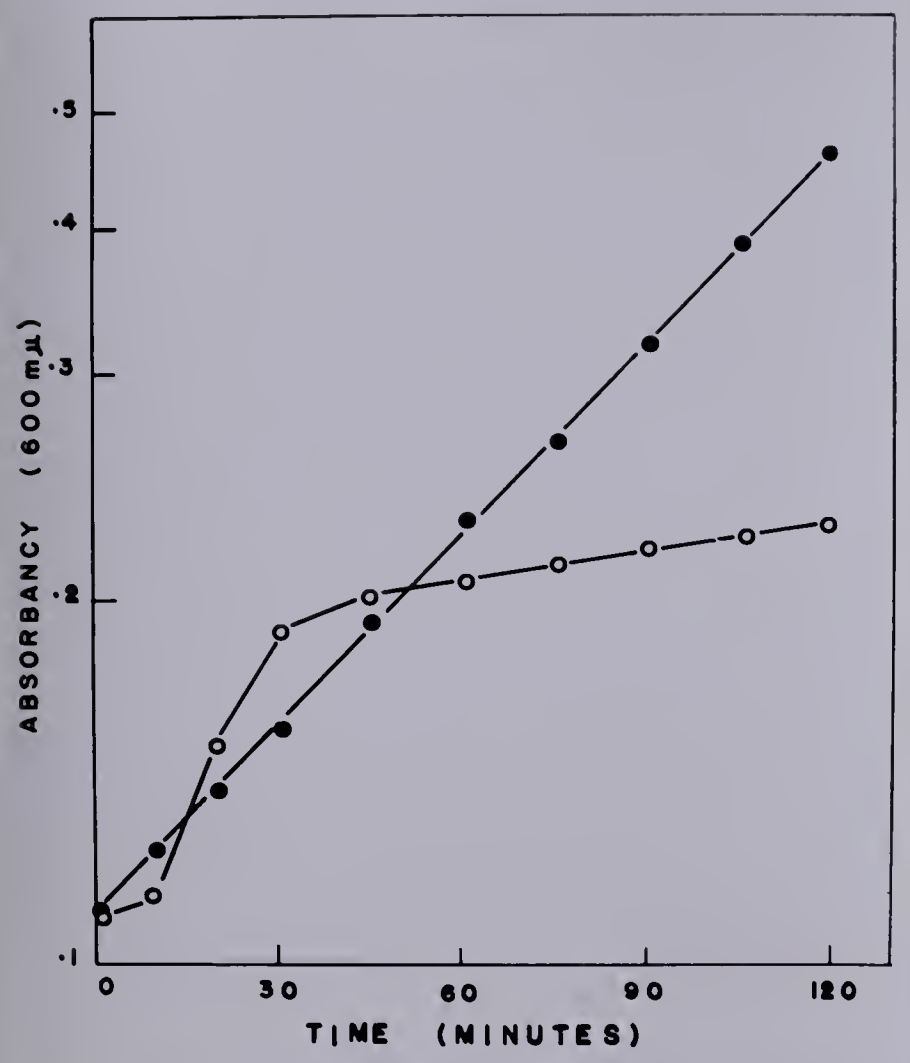
ABSORBANCY (600 mμ)
·5
·4
·3
·2
·1
0
30
60
90
120
TIME (MINUTES)

Figure 4. DNA Synthesis in Uninfected and Infected MAC-264 Cells.

Exponentially growing MAC-264 cells were infected with phage Ø-2 (M.I. = 7.5). Ten ml aliquots were taken for DNA determination by diphenylamine test.

● ——— ●, Control, uninfected;

O ——— O, Test, infected.

of DNA of the infected culture did not markedly increase after 45 minutes. The results are shown in Fig. 4.

b) RNA Synthesis:

The RNA synthesis in the infected cells increased slowly for 10 to 20 minutes after addition of Ø-2, and remained at the same level up to 120 minutes. As expected, the RNA in the uninfected cells increased logarithmically. Results are presented in Fig. 5.

c) Protein Synthesis:

There was a similar rate of protein synthesis in the infected cells as in the uninfected culture up to 60 minutes, but the rate of increase was reduced after that time. Results are shown in Fig. 6.

5. The One-Step Growth Curve of Phage Ø-2 in MAC-264 Cells

Multiplicities of infection ranging from 0.01 to 150 were used. The phage growth was measured by soft-agar layer method (Adams, 1959), and the intracellular growth was determined by opening the phage-infected bacteria by shaking the culture with a few drops of chloroform (Sechaud, and Kellenberger, 1956).

The growth curves, at all multiplicities used, showed a similar time relationship in respect to the eclipse period, the release period and also in respect to the burst size. Figs. 7, 8 and 9 show the one-step growth curves of the phage Ø-2 in infected MAC-264 cells with multiplicities of infection of 0.05, 5.3 and 100, respectively. From these curves, the eclipse period of this phage growth measures 30 minutes; latent period, 40 to 45 minutes; rise period, 30 to 35 minutes; and the burst size, 120 to 140 phage particles per infected cell.

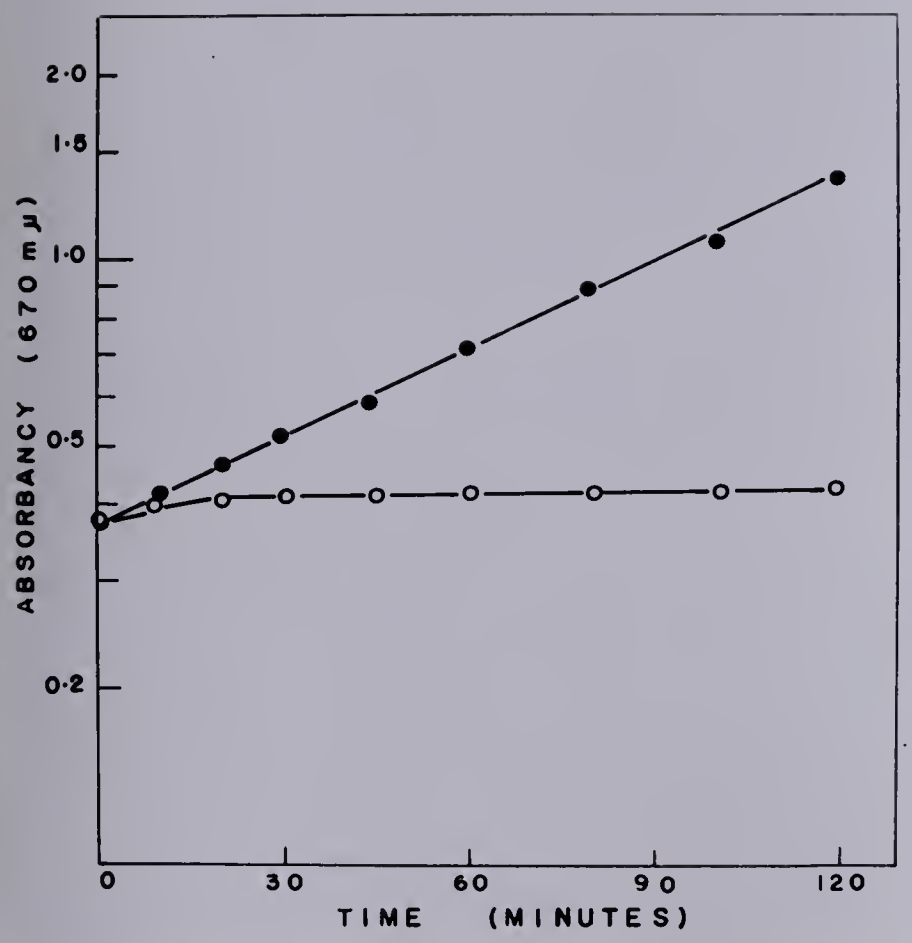
2·0
1·5
1·0
0·5
0·2
ABSORBANCY (670 mμ)
0
30
60
90
120
TIME (MINUTES)

Figure 5. RNA Synthesis in Uninfected and Infected MAC-264 Cells.

Exponentially growing MAC-264 cells were infected with phage Ø-2 (M.I.= 7.5). One ml aliquots were taken for RNA determination by orcinol test.

0 ——————— 0, Control, uninfected;

0 ——————— 0, Test, infected.

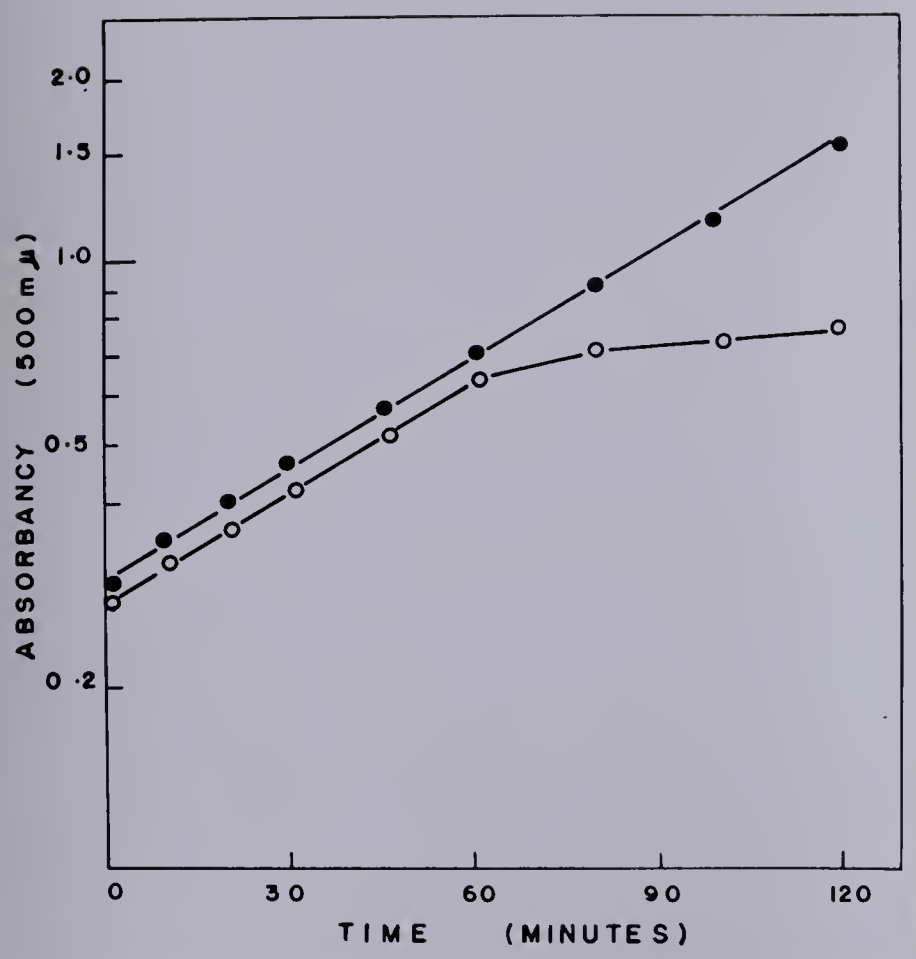
2·0
1·5
1·0
0·5
0·2
ABSORBANCY (500 mμ)
0
30
60
90
120
TIME (MINUTES)

Figure 6. Protein Synthesis in Uninfected and Infected MAC-264 Cells.

Exponentially growing MAC-264 cells were infected with phage Ø-2 (M.I.= 7.5). Aliquots were taken for protein determination by Lowry's method.

● ———— ●, Control, uninfected;

O ———— O, Test, infected.

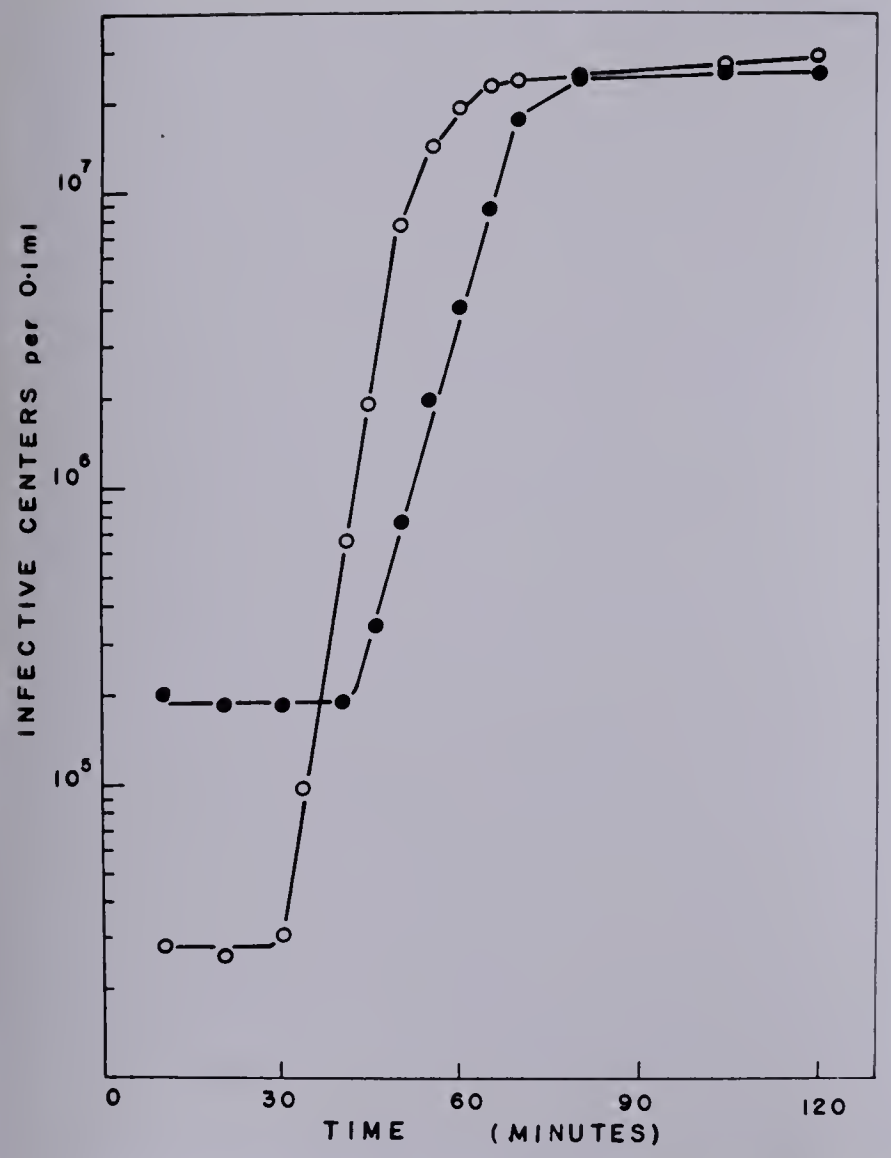
INFECTIVE CENTERS per 0·1 ml
10^7
10^6
10^5
0
30
60
90
120
TIME (MINUTES)

Figure 7. One-Step Growth Curve of Phage Ø-2 in P. aeruginosa Strain MAC-264 Cells. (M.I. = 0.05)

Two-tenth ml of phage Ø-2 concentrate was added to 1.8 ml MAC-264 cells in nutrient broth in a 37° water bath shaker with constant shaking. Aliquots were removed at time intervals, diluted and plated for determination of plaque forming units by soft-agar layer method.

● ——— ●, Extracellular;

O ——— O, Intracellular.

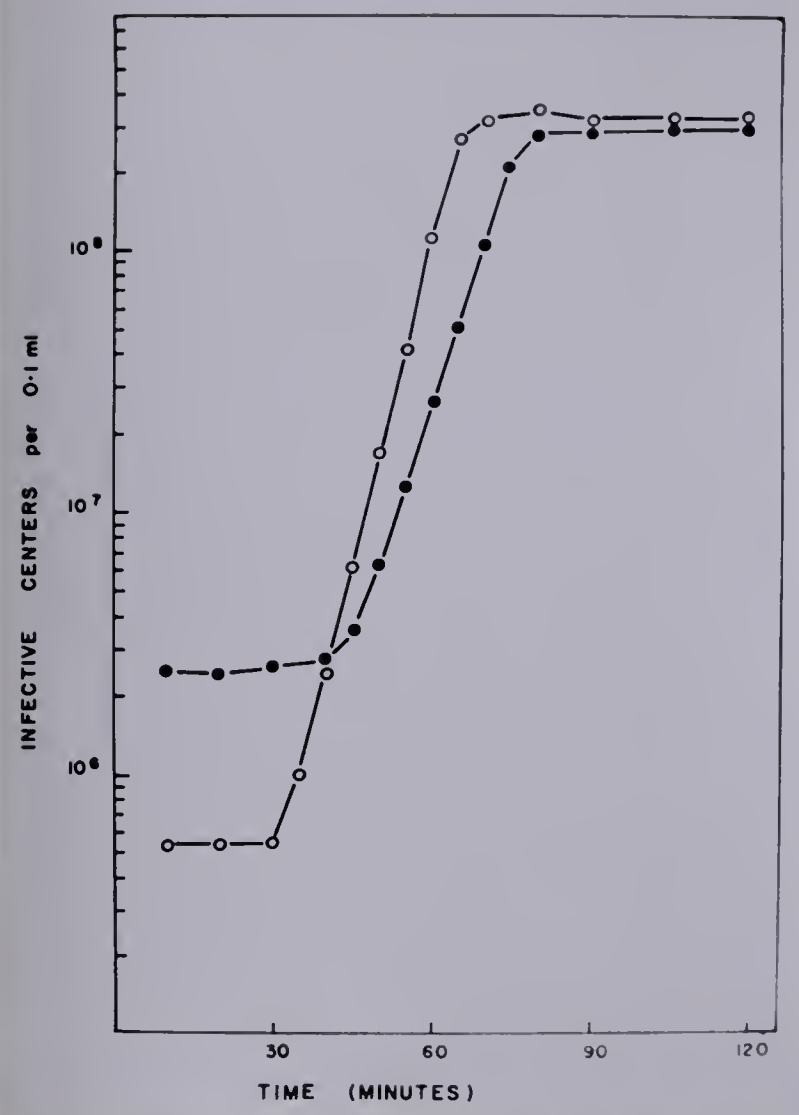
INFECTIVE CENTERS per 0·1 ml
10⁸
10⁷
10⁶
30
60
90
120
TIME (MINUTES)

Figure 8. One-Step Growth Curve of Phage Ø-2 in P. aeruginosa Strain MAC-264 Cells. (M.I. = 5.3)

For legend, see Fig. 7.

●———— ●, Extracellular;

0———— 0, Intracellular.

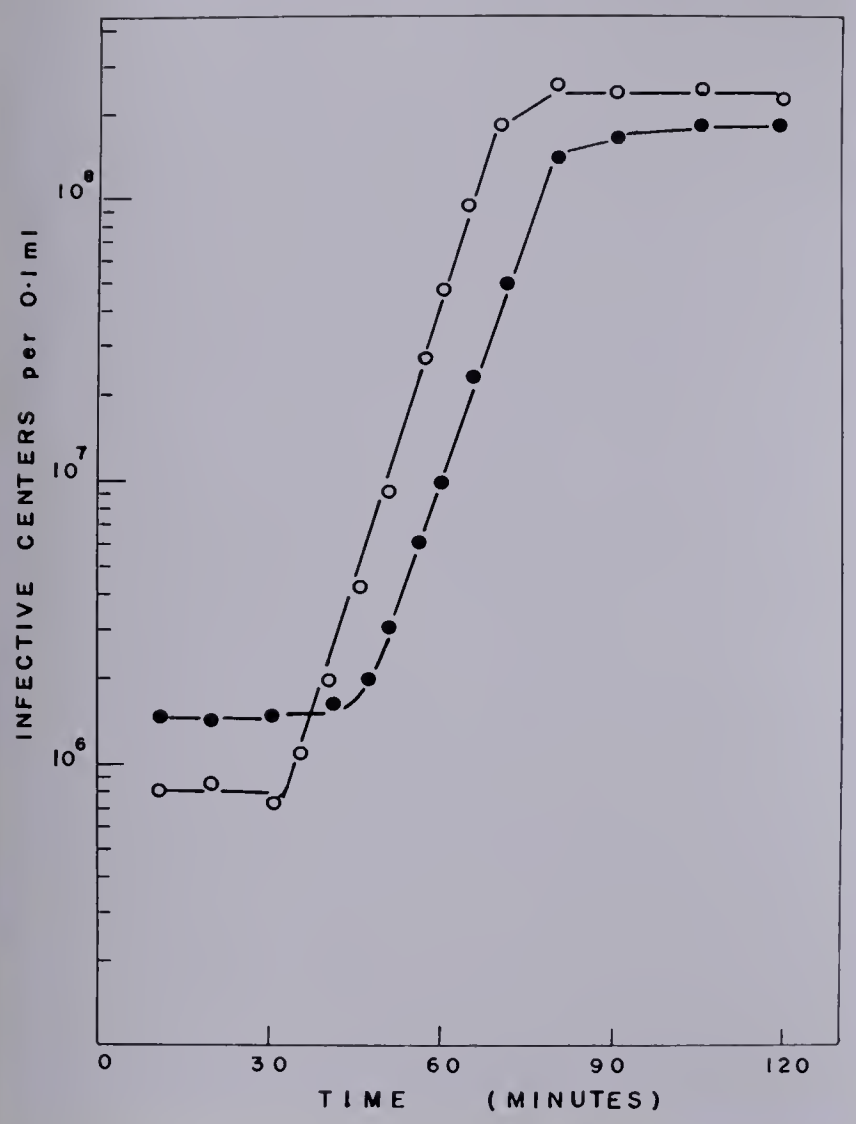
INFECTIVE CENTERS per 0·1 ml
10^8
10^7
10^6
0
30
60
90
120
TIME (MINUTES)

Figure 9. One-Step Growth Curve of Phage Ø-2 in P. aeruginosa Strain MAC-264 Cells. (M.I. = 100)

For legend, see Fig. 7.

● ——— ●, Extracellular;

○ ——— ○, Intracellular.

6. Effect of Chloramphenicol on Free Phage, Uninfected and Infected MAC-264 Cells

a) Effect on Free Phage:

Chloramphenicol (CM) was found to have neither activating nor inactivating effect on free phage Ø-2 in a range of concentrations of 10 to 1000 µg/ml.

b) Effect on Uninfected Cells:

When CM, at a final cencentration of 80 µg/ml, was added to an exponentially growing MAC-264 cells, 85% of them lost their colony forming ability in 15 minutes and remained at this level for the duration of this experiment (180 minutes). The removal of CM at 15, 30 and 50 minutes after addition, by diluting in warm medium to a level which had no more toxicity to the cells, allowed the surviving cells to resume growth at the same rate as the untreated cells. However, this resumption in growth was preceeded by a delay of about 50 minutes in each case. This indicates that the effect of CM in cell growth is reversible by its removal. The results are shown in Fig. 10.

c) Effect on Infected Cells:

The effect of CM in the concentration range of 20 to 120 µg/ml on the Ø-2 infected cells (M.I. = 8.2) was studied. CM was added to the infected cultures 10, 20 and 30 minutes after infection. Cencentrations lower than 60 µg/ml reduced the burst size, but did not stop phage replication. But when 80 to 120 µg/ml CM was added to the infected cultures 10 minutes after infection, no phage production could be measured and the infective centers decreased slowly. On the contrary, phage replication occurred, if CM was added 20 to 30 minutes after infection, but the burst size was smaller than that of the control cells (without CM).

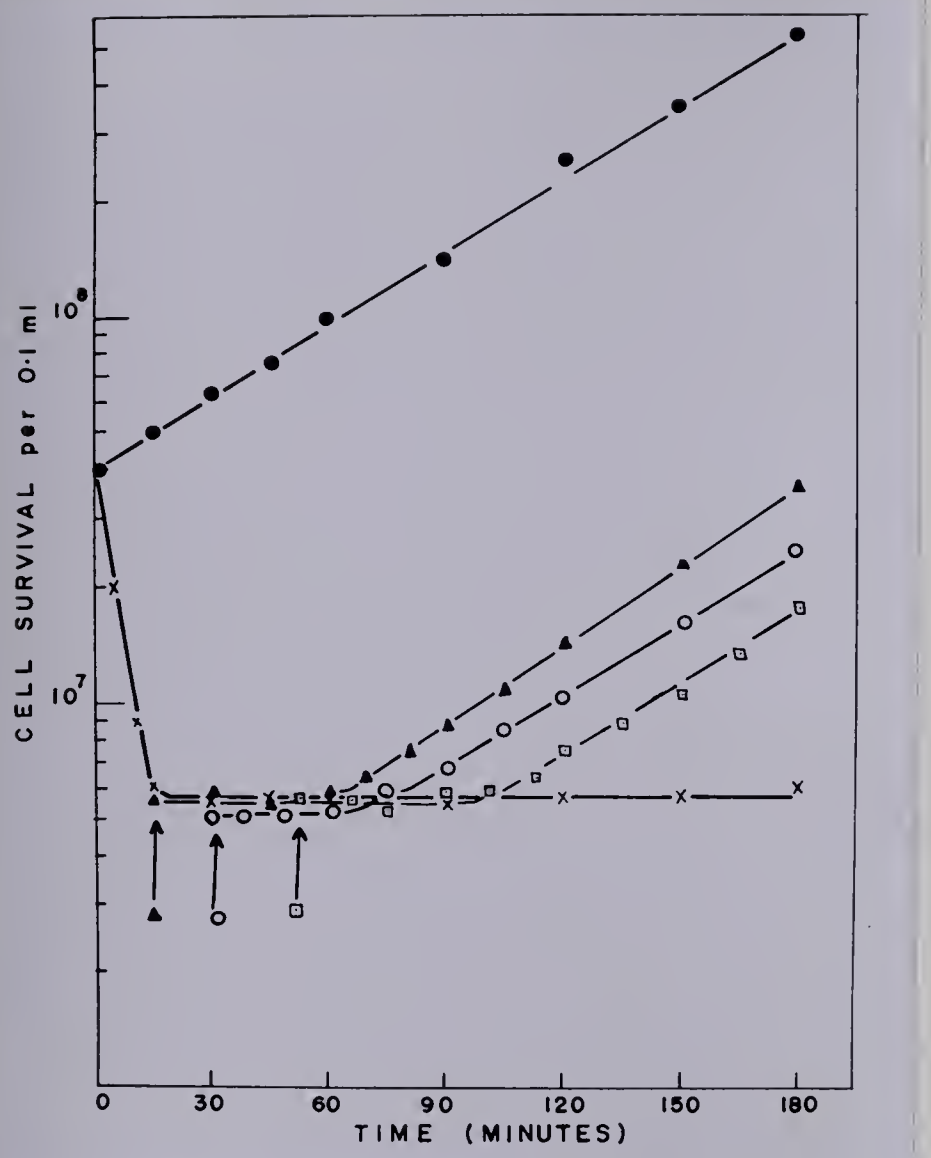
CELL SURVIVAL per 0·1 ml
10^8
10^7
0
30
60
90
120
150
180
TIME (MINUTES)

Figure 10. Effect of Chloramphenicol on MAC-264 Cells and Effect of Its Removal.

Chloramphenicol, at a final concentration of 80 µg/ml, was added to an exponentially growing MAC-264 culture at zero time.

After certain time intervals, chloramphenicol was removed by diluting in warm fresh nutrient broth. Cell survival was determined by plating 0.1 ml aliquots on nutrient agar plates, in duplicate.

● ———— ●, Control, no chlormphenicol;

▲ ———— ▲, CM removed at 15 minutes;

0 ———— 0, CM removed at 30 minutes;

◘ ———— ◘, CM removed at 50 minutes;

X ———— X, CM not removed.

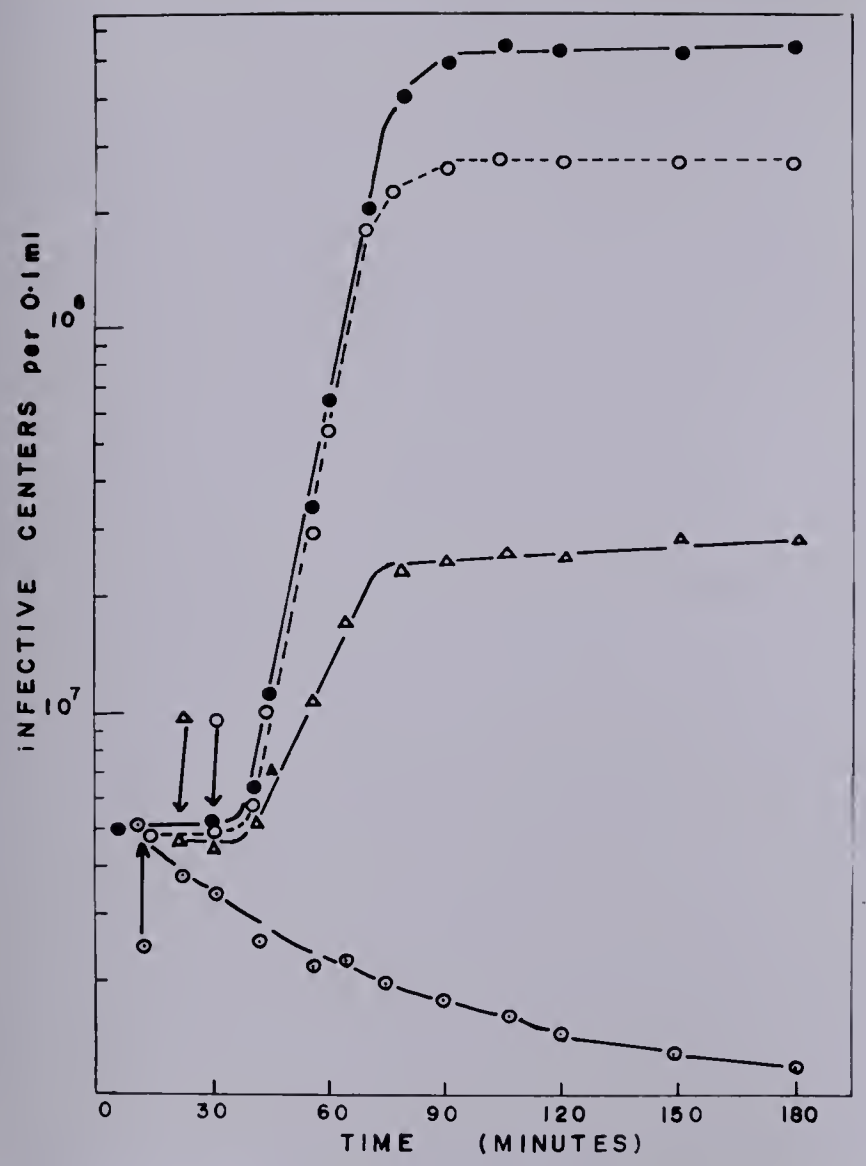
INFECTIVE CENTERS per 0·1 ml
10^8
10^7
0
30
60
90
120
150
180
TIME (MINUTES)

Figure 11. Effect of Chloramphenicol (CM) on Ø-2 Infected MAC-264 Cultures.

Chloramphenicol, at a final concentration of 80 µg/ml, was added to Ø-2 infected MAC-264 cultures (M.I. = 8.2) at different times after infection. Infective centers were determined by soft-agar layer method.

● ——— ●, Control, no CM;

0 ——— 0, CM added at 10 minutes;

△ ——— △, CM added at 20 minutes;

0 ——— 0, CM added at 30 minutes.

Results are shown in Fig. 11. The burst sizes, when CM was added at 20 and 30 minutes, were 6 and 48 respectively compared to 110 in the control culture. The differences in burst size after CM treatment reflect the amount of phage precursor material accumulated in the infected cells when CM was added. This was thoroughly investigated by Piechowski and Susman (1966 and 1967) who demonstrated that CM, as well as puromycin, inhibit phage protein synthesis within a few seconds, thus limiting the amount of phage precursor material available for maturation. The experiment shown in Fig. 11 demonstrates that, similarly to T_4, maturation of ∅-2 does not depend on concurrent protein synthesis.

The synthesis of DNA in the phage ∅-2 infected, CM-treated MAC-264 cells was studied. Exponentially growing MAC-264 cells were infected with ∅-2 at a M.I. of 8.5. CM, at a concentration of 80 μg/ml, was added to the infected culture 5, 10, 15 and 20 minutes after infection. Aliquots were removed from the cultures in order to determine the DNA content. No DNA synthesis was found when CM was added 5 minutes after infection. But addition at later times showed progressively less inhibitory action depending upon the time interval, and addition at 20 minutes showed no appreciable effect on DNA synthesis. The results are shown in Fig. 12.

7. Effect of Removal of Chloramphenicol from ∅-2 Infected MAC-264 Cultures

Both phage ∅-2 (M.I. = 9.5) and CM (80 μg/ml) were added to an exponentially growing MAC-264 culture. Thirty minutes later, the CM effect was removed by dilution.

The infected cells without CM showed a brust size of 128 while the infected cells in the presence of CM had a decrease in infective

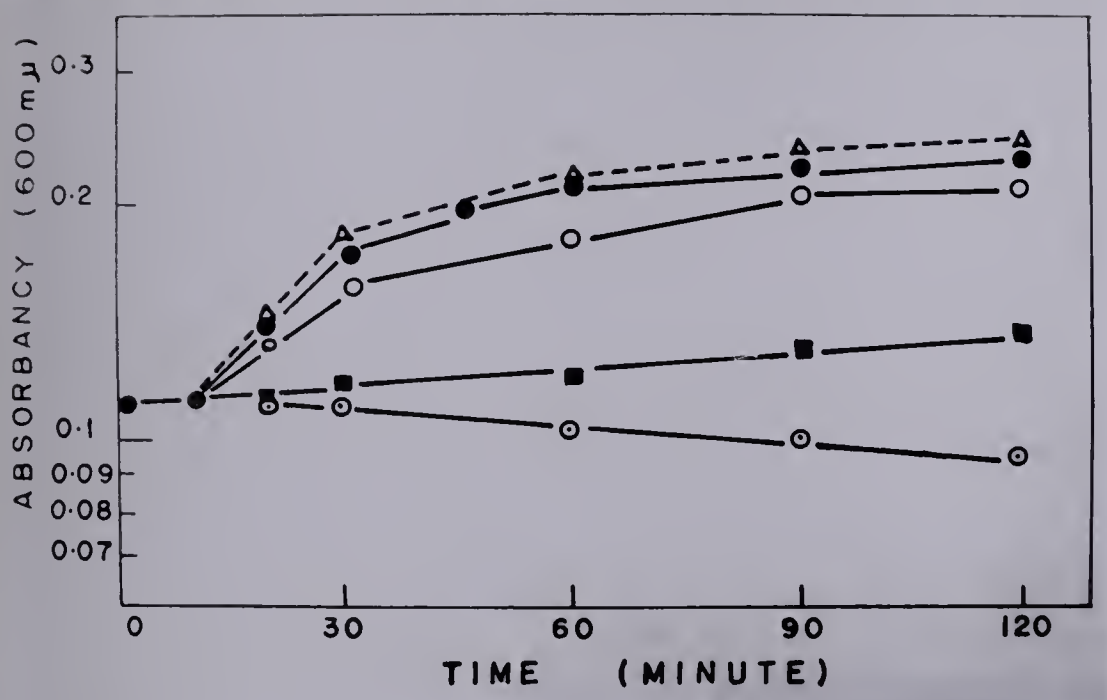
ABSORBANCY (600mμ)
0·3
0·2
0·1
0·09
0·08
0·07
0
30
60
90
120
TIME (MINUTE)

Figure 12. Effect of Chloramphenicol (CM) on the Synthesis of DNA by ϕ-2 Infected MAC-264 Cultures.

Chloramphenicol, at a final concentration of 80 µg/ml, was added to ϕ-2 infected MAC-264 cultures (M.I. = 8.5) at different times after infection. Ten ml aliquots were taken at intervals for determination of DNA content by diphenylamine test.

● ——— ●, Control, no CM;

0 ——— 0, CM added at 5 minutes;

■ ——— ■, CM added at 10 minutes;

0 ——— 0, CM added at 15 minutes;

△ ——— △, CM added at 20 minutes.

centres and no phage production. However, 50 minutes after removal of CM, phage production resumed but the burst size was smaller. This indicates that the effect of CM on phage replication is also reversible which is similar to the effect of it on uninfected cells described above (Section 6b). The results are presented in Fig. 13.

This experiment agrees with Ebisuzaki's report (1963) on the inhibition by tryptophan analogues of early protein synthesis in phage T_4 infected E. coli. After removal of the inhibitor (added at time 0), phage synthesis starts after a delay approximately equivalent to the exposure to the analogues. This shows that early phage functions precede the late functions by a strictly determined time interval.

8. Effect of Mitomycin C on Free Phage Ø-2, Uninfected and Infected MAC-264 Cells

a) Effect on Free Phage Ø-2:

Mitomycin C (MC) was found to have neither activating nor inactivating effect on free phage in the concentrations of 0.01 to 100 μg/ml of MC using a phage concentration of 1×10^{12} PFU/ml.

b) Effect on Uninfected Cells:

The method of determining MC effect on bacteria was described in Materials and Methods. The effect of various concentrations of MC on the colony forming ability of MAC-264 cells is shown in Fig. 14. Most of the cells promptly lost their colony forming ability after the addition of MC at concentrations of 1.0 to 10 μg/ml. A concentration of 0.1 μg/ml, or less, of MC showed no inhibitory effect.

MC at concentrations of 1.0 to 10 μg/ml was capable of inducing lysis of logarithmically growing MAC-264 culture 90 to 105 minutes

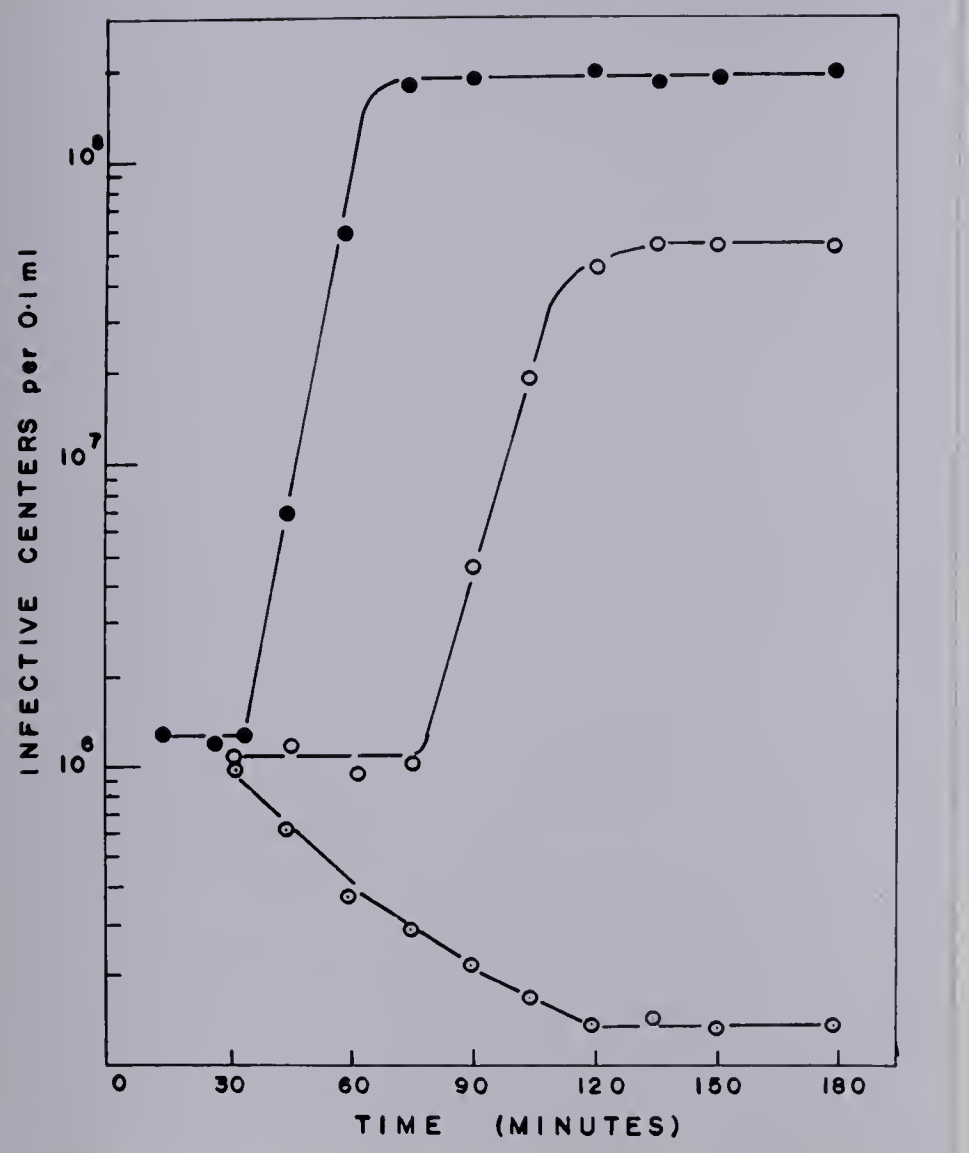
INFECTIVE CENTERS per 0·1 ml
10⁸
10⁷
10⁶
0
30
60
90
120
150
180
TIME (MINUTES)

Figure 13. Effect of the Removal of Chloramphenicol from ϕ-2 Infected MAC-264 Cells.

Both phage ϕ-2 (M.I. = 9.5) and CM (80 μg/ml) were added to an exponentially growing MAC-264 culture. Thirty minutes later, CM was removed from the culture by diluting in fresh warm nutrient broth. Infective centers were determined by soft agar layer method.

● ———— ●, Control, no CM;

0 ———— 0, CM removed at 30 minutes after infection;

0 ———— 0, CM not removed.

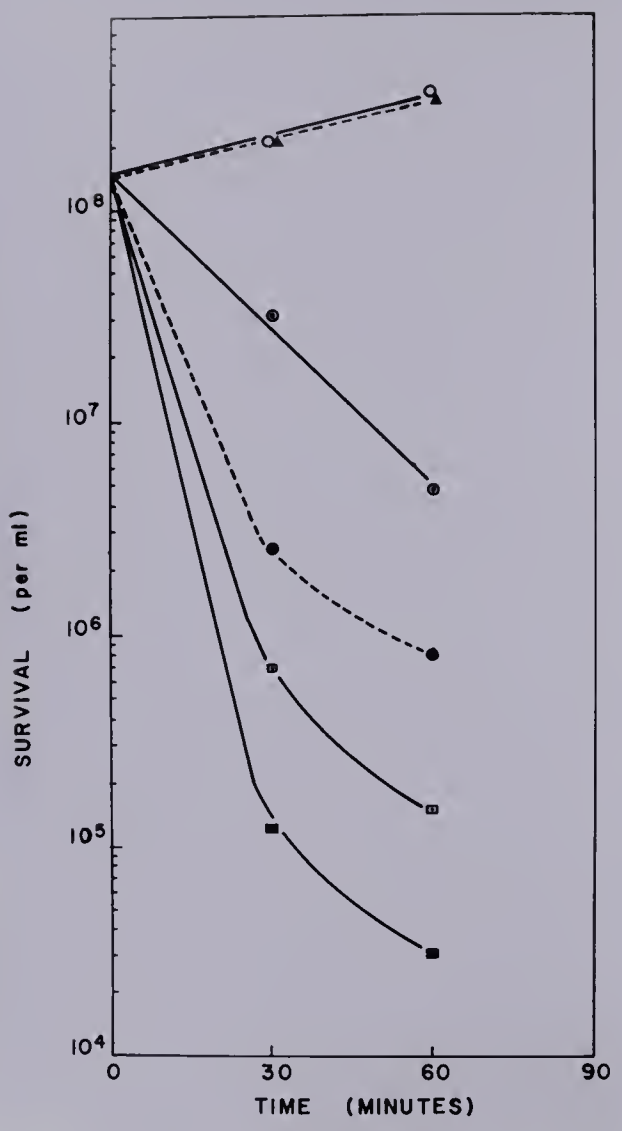
SURVIVAL (per ml)
10^8
10^7
10^6
10^5
10^4
0
30
60
90
TIME (MINUTES)

Figure 14. Survival of P. aeruginosa Strain MAC-264 after Treatment with Mitomycin C (MC).

Various concentrations of MC was added to exponentially growing MAC-264 cultures at zero time. One-tenth ml aliquots were removed and plated for colony forming capacity.

0 ———— 0, Control, no MC;

▲ ———— ▲, 0.01 and 0.1 µg/ml of MC;

◎ ———— ◎, 1 µg/ml MC;

● ———— ●, 2.5 µg/ml MC;

□ ———— □, 5 µg/ml MC;

■ ———— ■, 10 µg/ml MC.

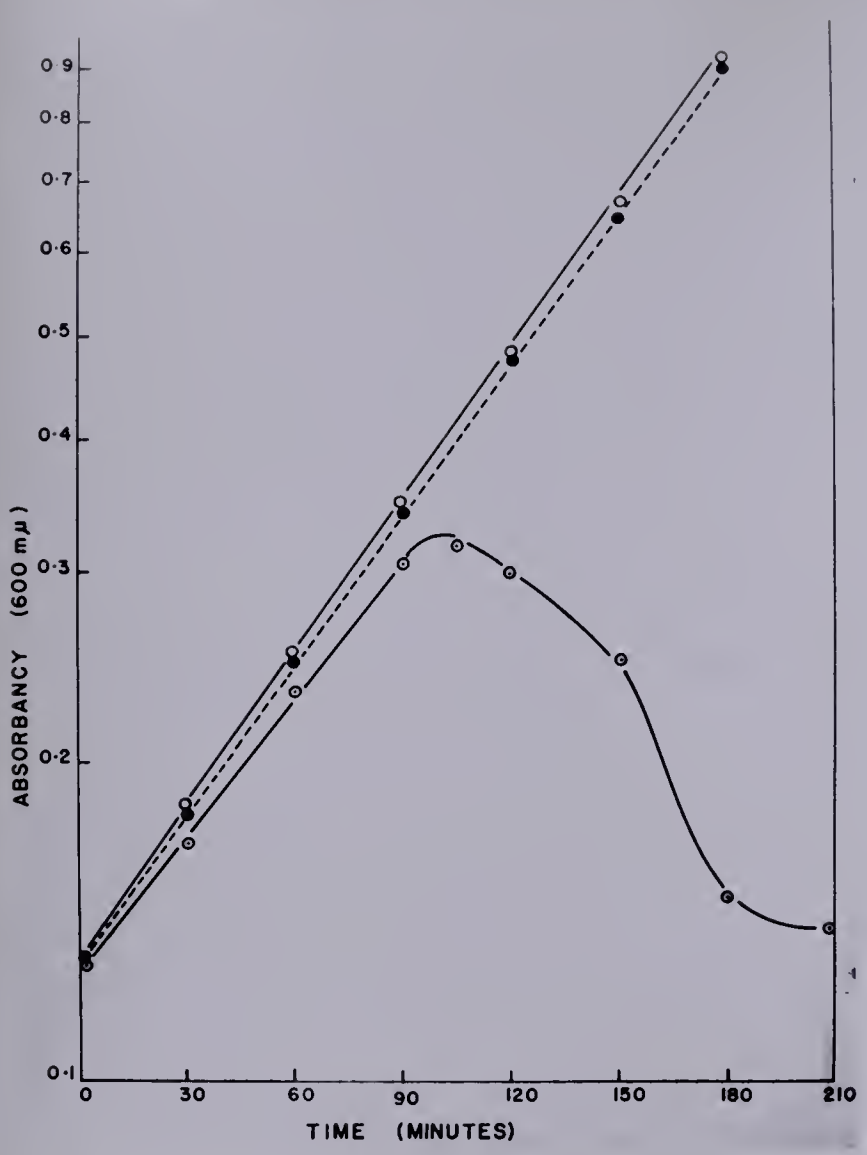
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
ABSORBANCY (600 mμ)
0
30
60
90
120
150
180
210
TIME (MINUTES)

Figure 15. Effect of Mitomycin C on Growth of MAC-264 Cultures.

Mitomycin C was added at zero time to MAC-264 culutres growing at 37° with constant shaking in nutrient broth. The optical density of the cultures was determined at 600 mμ in a Bausch and Lomb Spectronic 20 every 30 minutes.

0 ——— 0, Control, no MC;

● ——— ●, 0.01 to 0.1 μg/ml MC;

0 ——— 0, 1 to 10 μg/ml MC.

after addition as shown by the decrease in optical density. The results are presented in Fig 15. However, lower concentrations (0.01 to 0.1 μg/ml) of MC did not show such an effect.

c) Effect on Infected Cells:

An exponentially growing MAC-264 culture was treated wtih MC (0.1 to 10 μg/ml) and infected with ϕ-2 (M.I. = 7.5) at the same time. A normal growth curve of the phage, with respect to time and number of phage released from the infected cells, was obtained. This is shown in Fig. 16. (Compare with Figs. 7, 8 and 9 for ϕ-2 infected cells without MC-treatment).

9. Growth Curve of Phage ϕ-2 in MAC-264 Cells Previously Treated with MC

MC at a concentration of 2.5 μg/ml was added into an exponentially growing MAC-264 culture. The culture was incubated at 37° with shaking. Thirty, 60 and 90 minutes after addition of MC, 1.8 ml of the cells were infected with 0.2 ml ϕ-2 preparation at a M.I. of 9. There was no marked difference in latent period, rise period or burst size of the phage if the infection was 30 or 60 minutes after MC-treatment. If the culture was infected 90 minutes after addition of MC, a lower burst size was observed. The results are shown in Fig. 17.

10. Effect of MC on DNA, RNA, and Protein Synthesis in MAC-264 Cells

MC was added to an exponentially growing MAC-264 culture at a concentration of 2.5 μg/ml. Aliquots were removed every 15 minutes to determine the DNA, RNA and protein content by the procedures described in Materials and Methods.

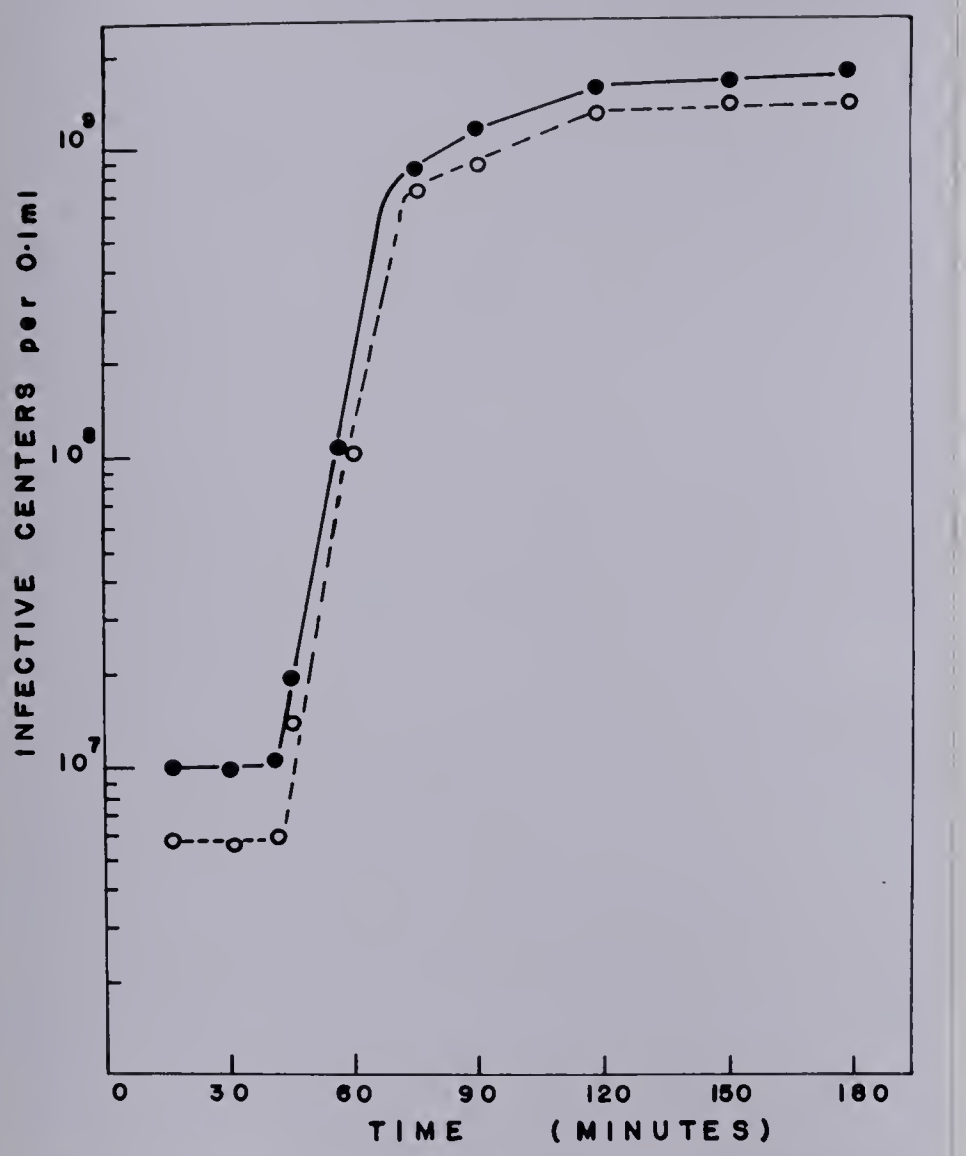
INFECTIVE CENTERS per 0·1ml
10^9
10^8
10^7
0
30
60
90
120
150
180
TIME (MINUTES)

Figure 16. Effect of Mitomycin on ϕ-2 Infected MAC-264 Cultures.

Both ϕ-2 (M.I. = 7.5) and MC (0.1 to 10 μg/ml) were added to an exponentially growing MAC-264 culture in a 37° water bath shaker. Infective centers were determined by the soft agar layer method.

● ——— ●, Control, no MC;

O -------- O, Test, with MC.

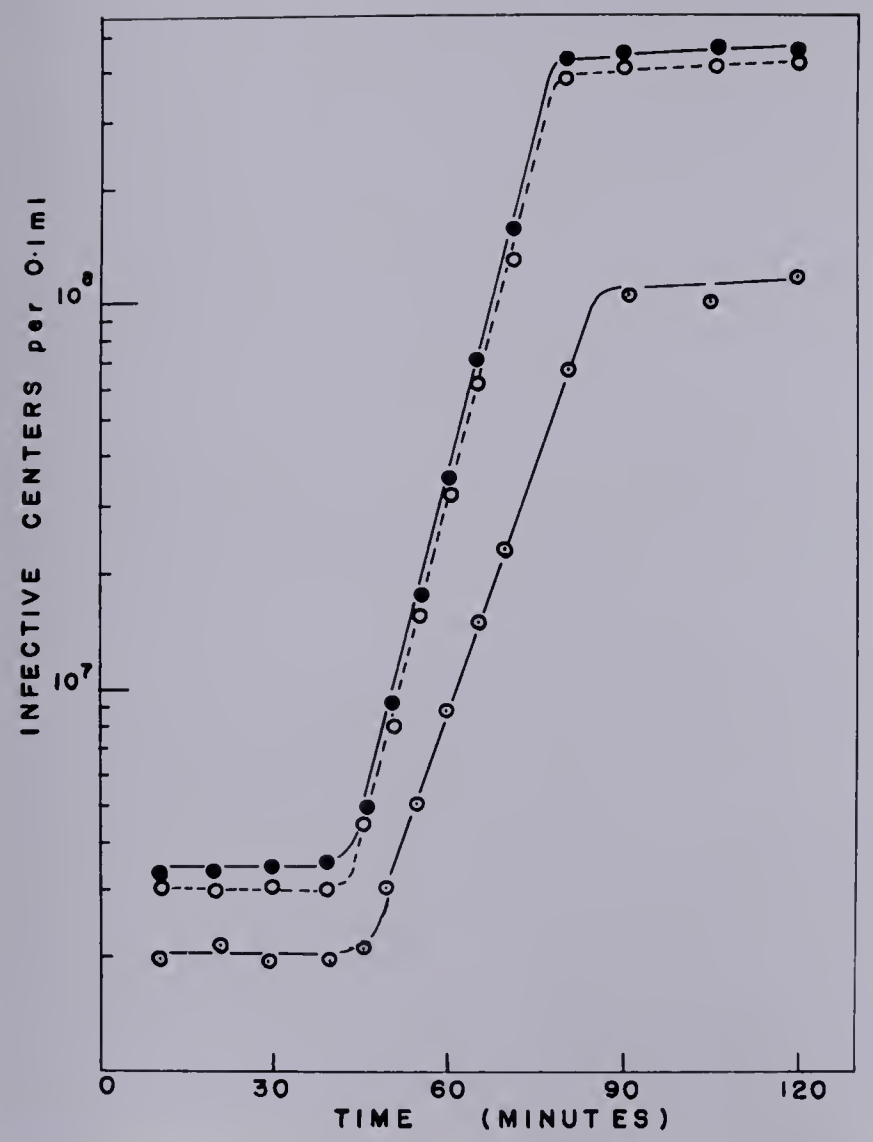
INFECTIVE CENTERS per 0·1ml
10^8
10^7
0
30
60
90
120
TIME (MINUTES)

Figure 17. Phage ϕ-2 Growth in MAC-264 Cells Previously Treated with Mitomycin C (MC).

MC at a concentration of 2.5 µg/ml was added to an exponentially growing MAC-264 culture. Thirty, 60 and 90 minutes after addition of MC, 1.8 ml of the culture were removed and infected with ϕ-2 at a M.I. = 9.0.

Infective centers were determined by the soft-agar layer method.

● ——— ●, Control, no MC;

0 -------- 0, Cells infected 30 and 60 minutes after MC-treatment;

0 ——— 0, Cells infected 90 minutes after MC-treatment.

a) DNA Synthesis:

Previous reports pointed out that the primary effect of MC was the inhibition of DNA synthesis in cells (Pricer and Weissbach, 1964a, 1964b; Sekiguchi and Takagi, 1960; Suzuki and Kilgore, 1967). This study was undertaken to determine the effect of MC on DNA synthesis in MAC-264 cells. The DNA in control untreated cells increased logarithmically, as expected. In contrast, the DNA synthesis of the MC-treated culture stopped immediately upon addition of MC and the level remained stationary for a period of 45 minutes. This period was then followed by a sudden increase. (Fig. 18). This resumption in DNA synthesis indicated that phage replication was probably taking place, since MAC-264 cells were thought to be lysogenic and since the temperate phage inducing ability of MC was reported (Korn and Weissbach, 1962; Otsuji et al., 1959; Levine, 1961; Endo et al., 1965; Sutton and Quadling, 1963; Seaman et al., 1964; Welker and Campbell, 1965).

b) RNA and Protein Synthesis:

Fig. 19 shows that both RNA and protein synthesis were not markedly affected until 60 to 75 minutes respectively after addition of MC. After this time they both remained at a constant level.

c) Induction of Cells in Nutrient Broth:

Since DNA synthesis in MC-treated cells resumed after 45 minutes, the culture was examined further for the induction of either temperate phages or pyocins. Cells grown in nutrient broth were induced by 2.5 μg/ml of MC. Lysis occurred 90 to 105 minutes after exposure, and examination of the lysates with an electron microscope revealed the presence of a short-tailed phage. To this phage the designation Ø-MC was given.

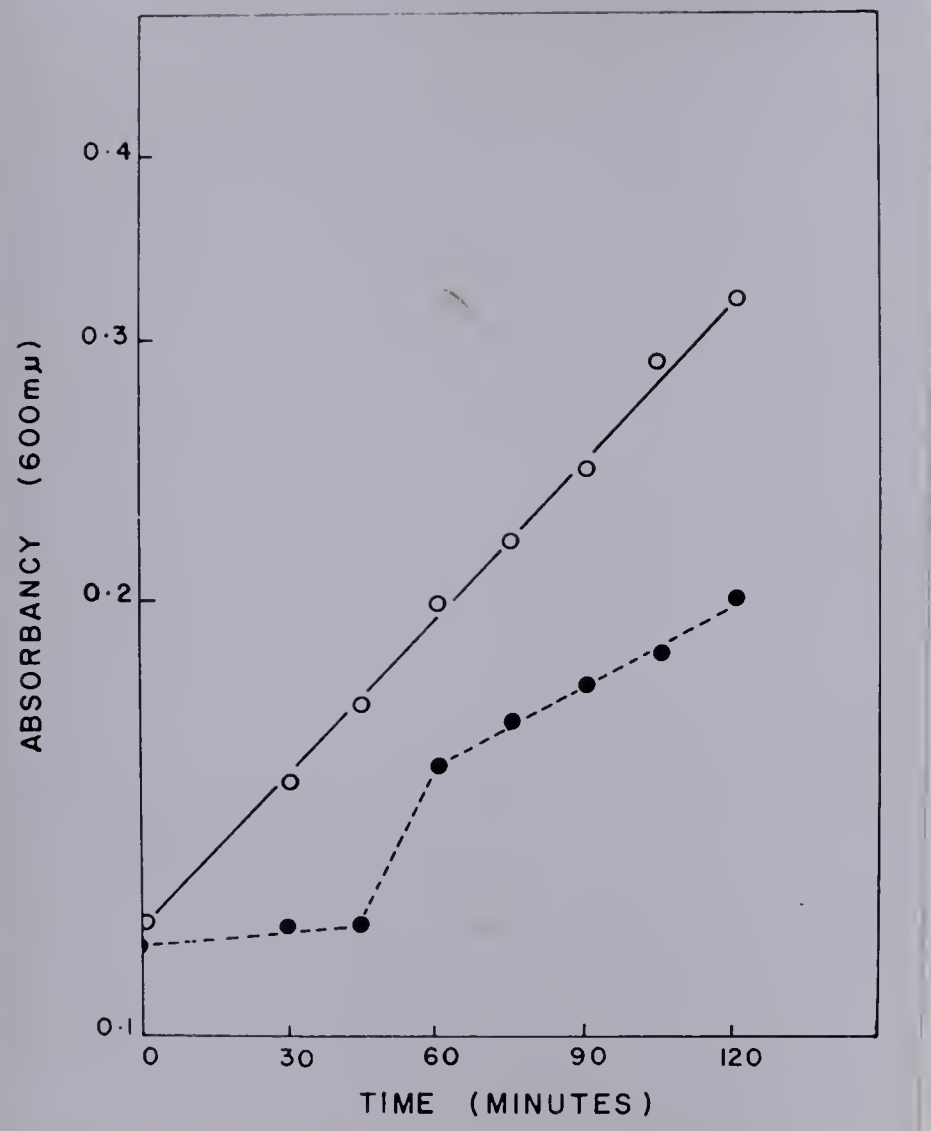
0·4
0·3
0·2
0·1
ABSORBANCY (600mμ)
0
30
60
90
120
TIME (MINUTES)

Figure 18. Effect of Mitomycin C (MC) on DNA Synthesis in MAC-264 Cells.

MC (2.5 µg/ml) was added to an exponentially growing MAC-264 culture in a 37° water bath shaker.

Ten ml aliquots were taken at intervals to determine DNA contents by the diphenylamine test.

O ———— O, Control, no MC;

● -------- ●, Test, with MC.

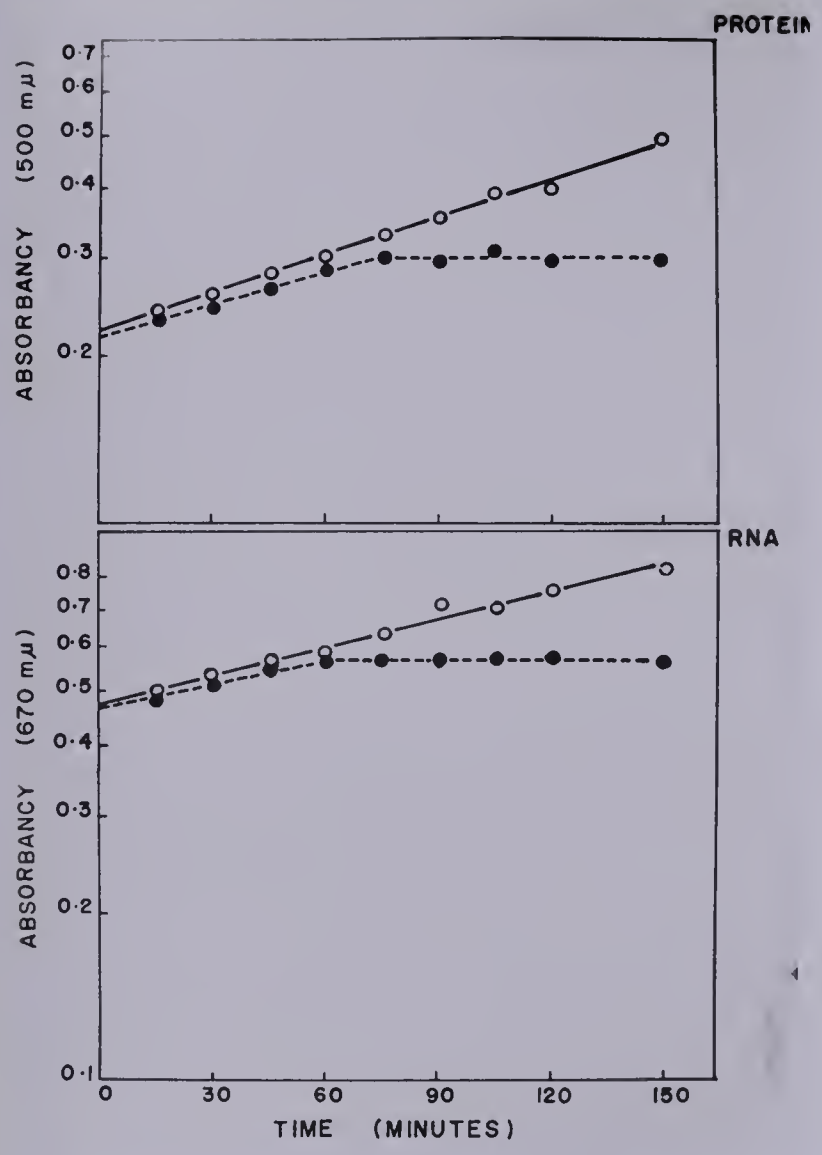
PROTEIN
ABSORBANCY (500 mμ)
0·7
0·6
0·5
0·4
0·3
0·2
RNA
ABSORBANCY (670 mμ)
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
30
60
90
120
150
TIME (MINUTES)

Figure 19. Effect of Mitomycin C (MC) on RNA and Protein Synthesis in MAC-264 Cells.

MC at a final concentration of 2.5 μg/ml was added to cultures growing at 37° with constant shaking in nutrient broth at zerio time. RNA and protein were measured by the orcinol and Folin phenol reagents respectively.

O ——— O, Control, no MC;

● -------- ●, Test, with MC.

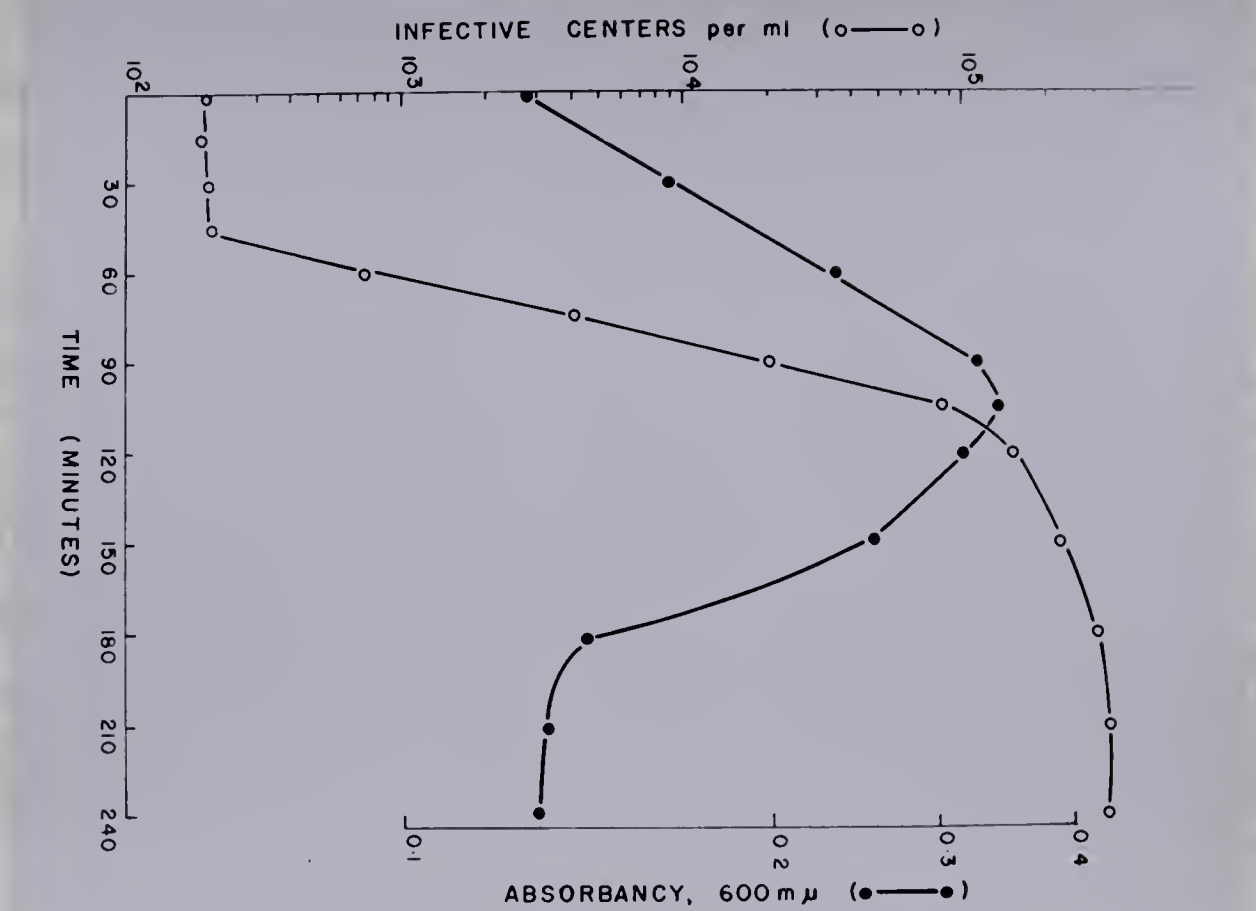
INFECTIVE CENTERS per ml (o——o)
10²
10³
10⁴
10⁵
TIME (MINUTES)
30
60
90
120
150
180
210
240
0·1
0·2
0·3
0·4
ABSORBANCY, 600 mμ (●——●)

Figure 20. Development of Infective Centers in Mitomycin C (MC) Treated Cultures of P. aeruginosa Strain MAC-264.

MC (2.5 µg/ml) was added to an exponentially growing MAC-264 culture at zero time. Infective centers were determined by soft-agar layer method using P. aeruginosa strain PAE-2-1 as the indicator. Changes in optical density of the cultures were also determined for the corresponding times.

O ———— O, Infective centers;

● -------- ●, Optical density.

Forty-two strains of P. aeruginosa and fluorescens were tested for their ability to form plaques upon the addition of MC-treated cells. P. aeruginosa strain PAE-2-1 was found to be the only sensitive indicator for this induced temperate phage, Ø-MC. The plaques produced on strain PAE-2-1 were distinct and minute, measured about 0.2 to 0.3 mm in diameter. The production of Ø-MC infective units from induced cultures of MAC-264 growing in nutrient broth using PAE-2-1 as the indicator strain is shown in Fig. 20. Increased phage production was measurable approximately 30 minutes before a detectable change, and subsequent decrease, in optical density.

12. Electron Microscopic Study of Phage Ø-MC Induced by MC in P. aeruginosa Strain MAC-264 Cells

To an exponentially growing MAC-264 culture, 2.5 μg/ml MC was added, and the culture incubated in a 37° water-bath shaker for a further 150 minutes. Ten ml aliquots of the cells were removed at intervals during this period, fixed by the Ryter-Kellenberger technique, sectioned and stained with uranyl acetate and lead citrate. Fig. 21 shows the cell morphology just before addition of MC. The plasma membrane and the sinuous cell wall are readily discernable. This is a common feature of Gram-negative organisms (van Iterson, 1965). The centrally located DNA region is characterized by the lighter part. The black deposits seen in the DNA region are not artifacts resulting from uranyl treatment as suggested by Fuhs (1965), but are characteristic structures in this strain (Yamamoto, 1967). They increase in size after MC-treatment and may possibly be the sites of rhapidosomes.

Figures 22 and 23 show the cells 30 to 60 minutes after addition of MC, respectively. There is no obvious change inside the cells, but numerous

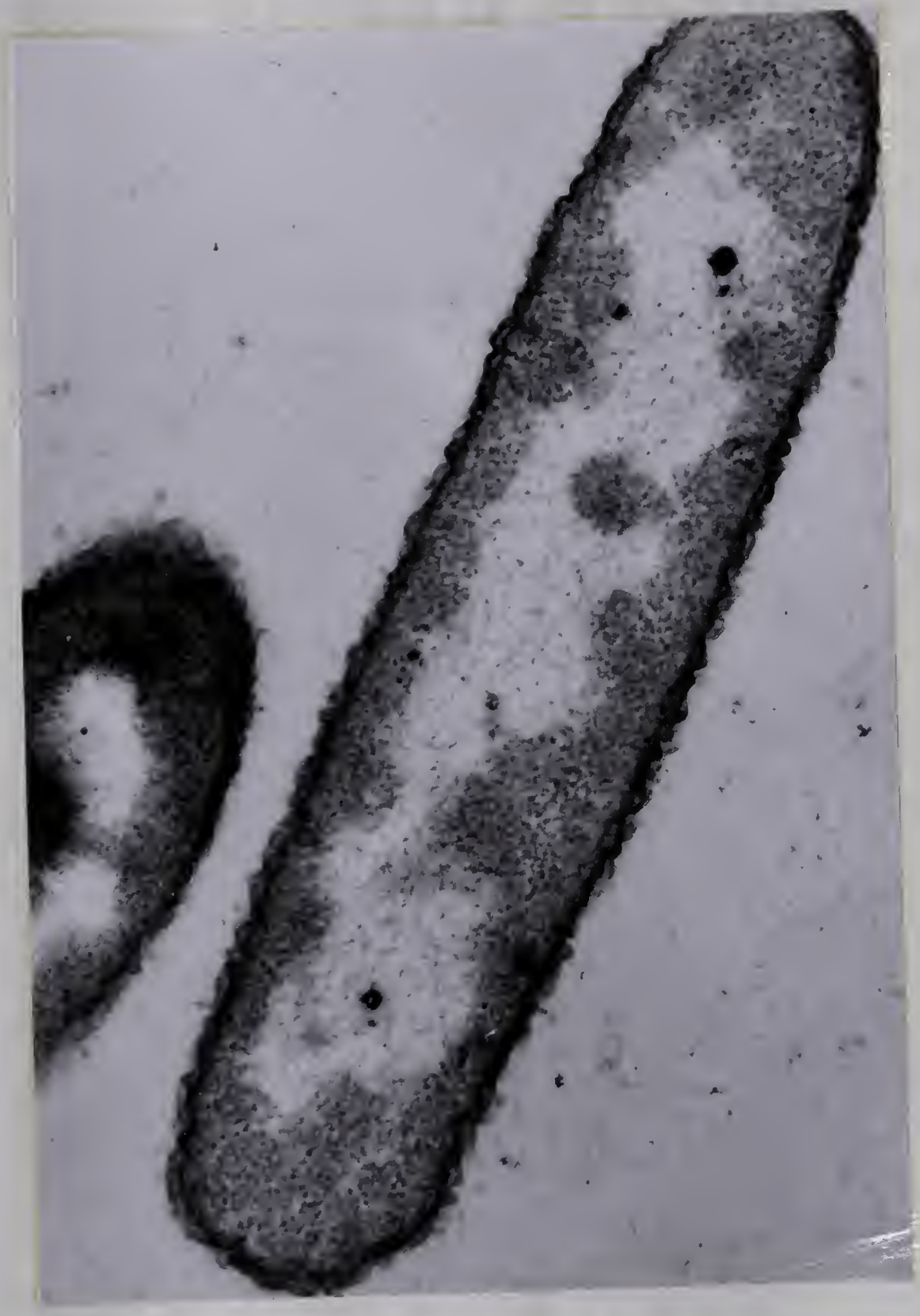

Figure 21. Electron Micrograph of Normal Pseudomonas aeruginosa Strain MAC-264 Cells

Cells grown in nutrient broth in a 37° water bath shaker, fixed by Ryter-Kellenberger technique, embedded and sectioned as described in Materials and Methods. X 50,000.

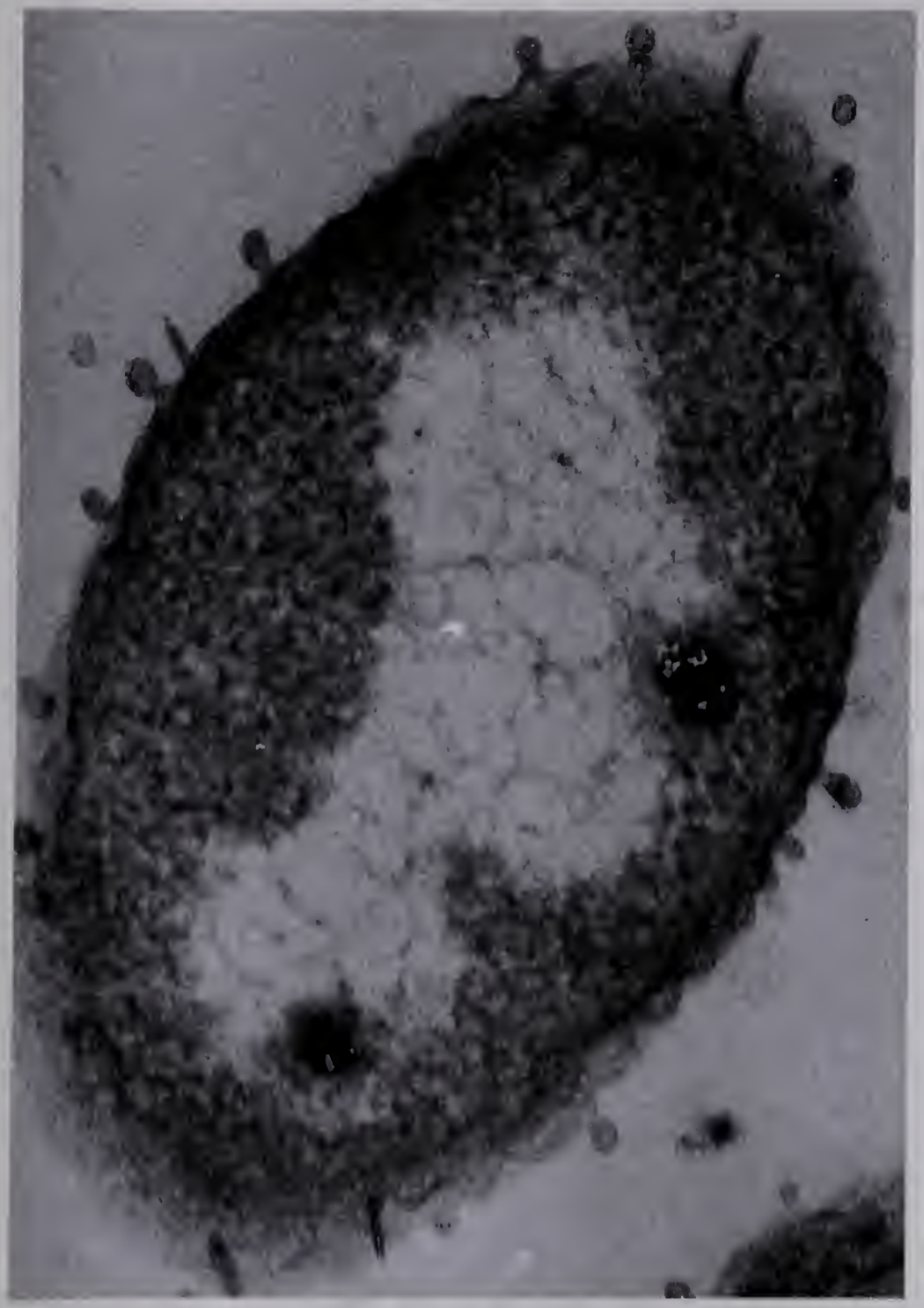

Figure 22. Electron Micrograph of MAC-264 Cells 30 minutes after Mitomycin C Treatment.

Mitomycin C at a concentration of 2.5 µg/ml was added to an exponentially growing MAC-264 culture. Thirty minutes later, 10 ml of the bacterial culture were removed and fixed by the Ryter-Kellenberger technique, embedded and sectioned as described in Materials and Methods. X 100,000.

Figure 23. Electron Micrograph of MAC-264 Cells 60 Minutes after Mitomycin C Treatment.

For legend, see Fig. 22.

X 85,000.

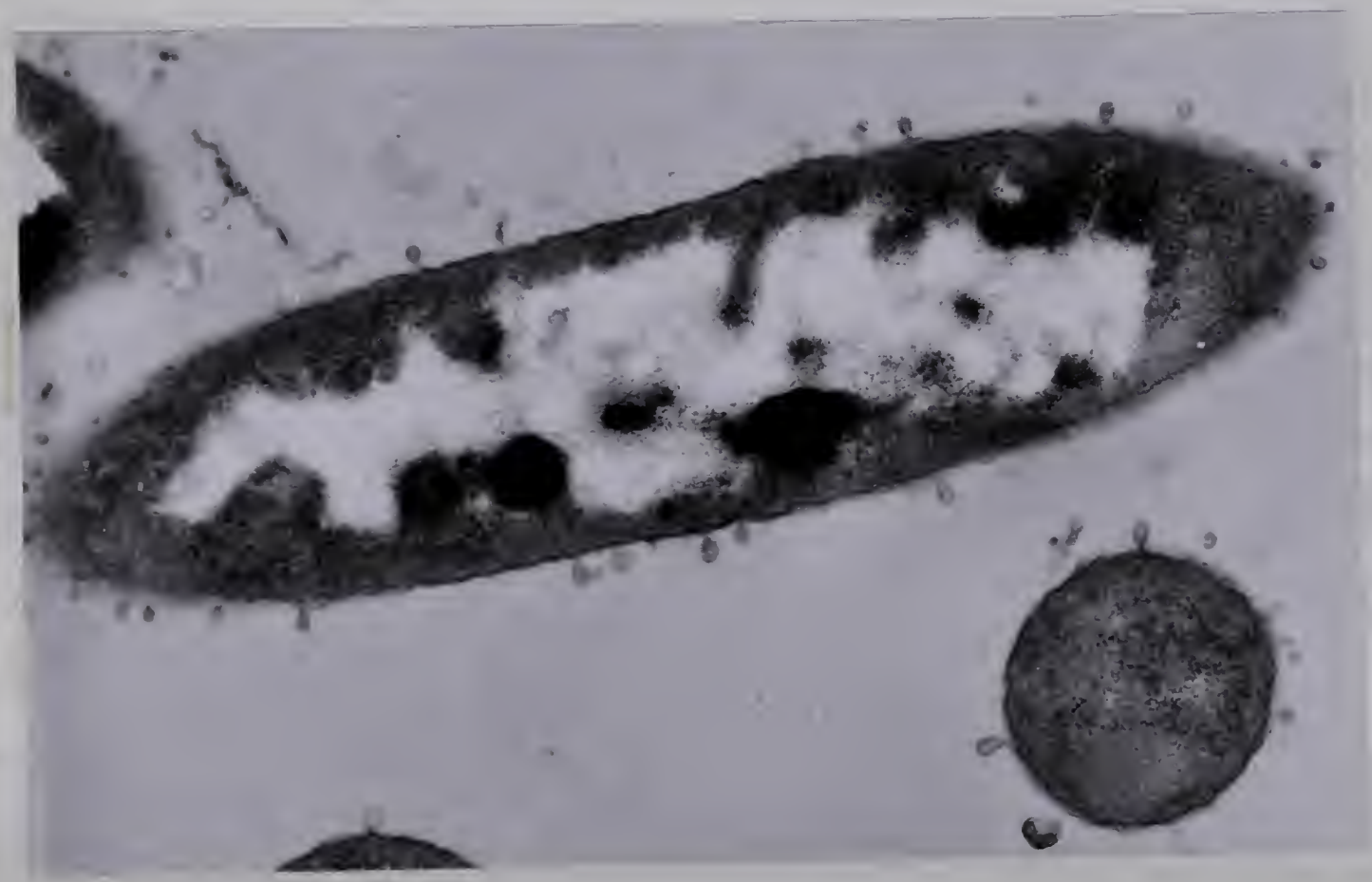

Figure 24. Electron Micrograph of MAC-264 Cells 75 Minutes after Mitomycin C Treatment.

For legend, see Fig. 22.

X 40,000.

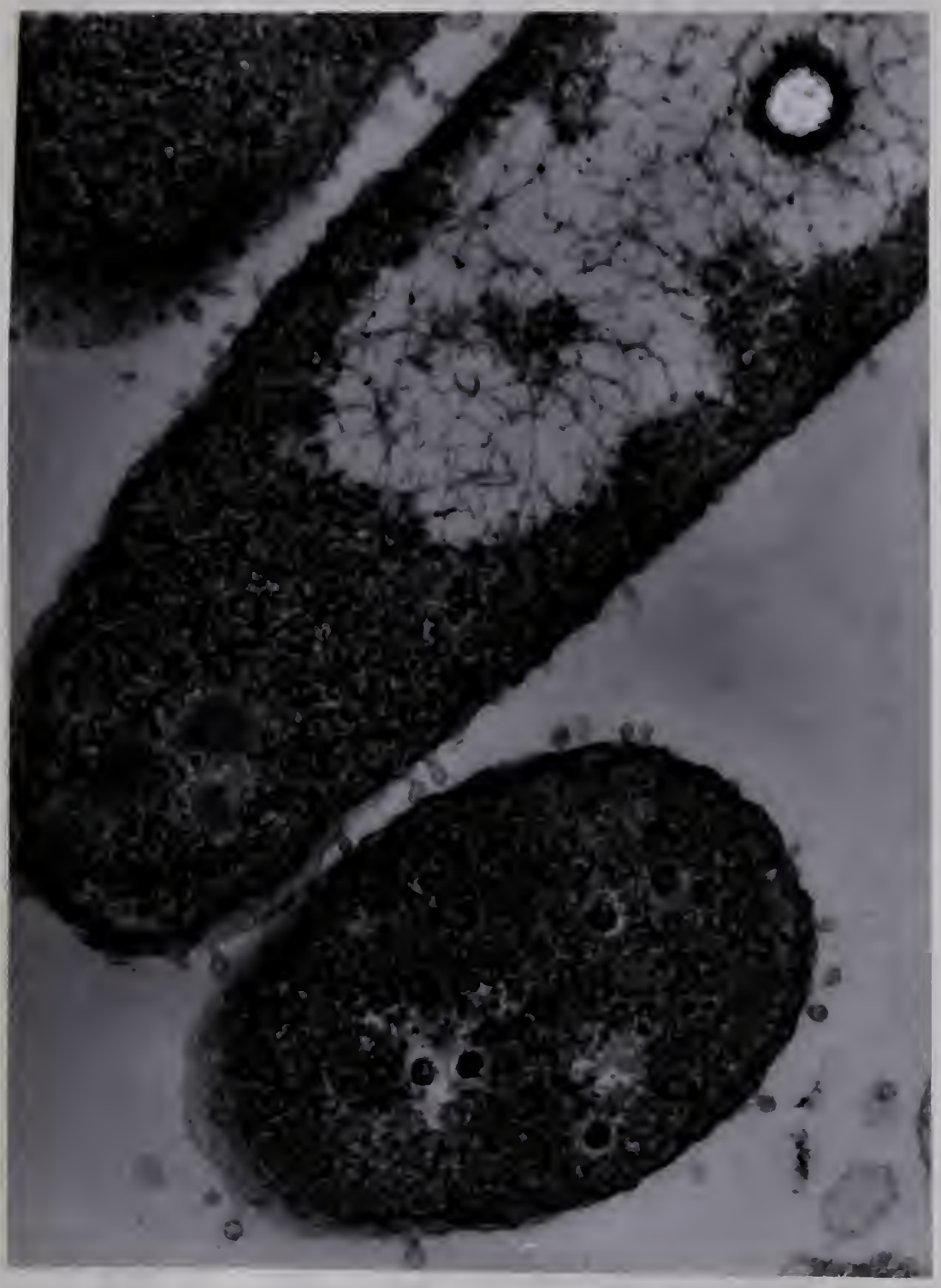

Figure 25. Electron Micrograph of MAC-264 Cells 90 Minutes after Mitomycin C Treatment.

For legend, see Fig. 22.

X 70,000.

papillary structures can be seen along the cell wall. They seem to be continuous with the cell membrane and have characteristic double-layered outline.

Cells 75 minutes after MC-treatment, as shown in Fig. 24, still have their normal appearance except at the tips of some cells, where less granular, homogeneous areas can be observed. These areas are thought to be the regions of phage precursor pool.

Phages begin to appear 90 minutes after exposure to MC, and the homogeneous areas are more obvious at the ends of the cells. This is shown in Fig. 25.

More phages were observed at later times. They are shown in Figs. 26, 27, 28 and 29. The induction of this phage has a feature that most of the phage particles are synthesized at the ends of the cells, and the host nucleoplasm appeared normal even when numerous phage particles were produced inside them. Cells observed to be in various stages of division were also induced and showed characteristic phage replication as shown in Fig. 29.

13. Cesium Chloride Equilibrium Centrifugation of MC-treated MAC-264 Cell Lysates and of Phage Ø-MC

An analysis of the MAC-264 cell lysates 180 minutes after addition of MC is presented in Fig. 30. Two major components were identified by absorbancy measurement: banding at buoyant densities of 1.468 and 1.305 g/ml, respectively. The material banding at ρ = 1.468 had a very high titer of infectivity when plated on P. aeruginosa strain PAE-2-1 while the material of ρ = 1.305 did not show any infectivity. Purified Ø-MC grown on PAE-2-1 cells showed a very close density (1.470 g/ml) in CsCl when centrifuged to equilibrium at the same time. The band at ρ = 1.305 g/ml

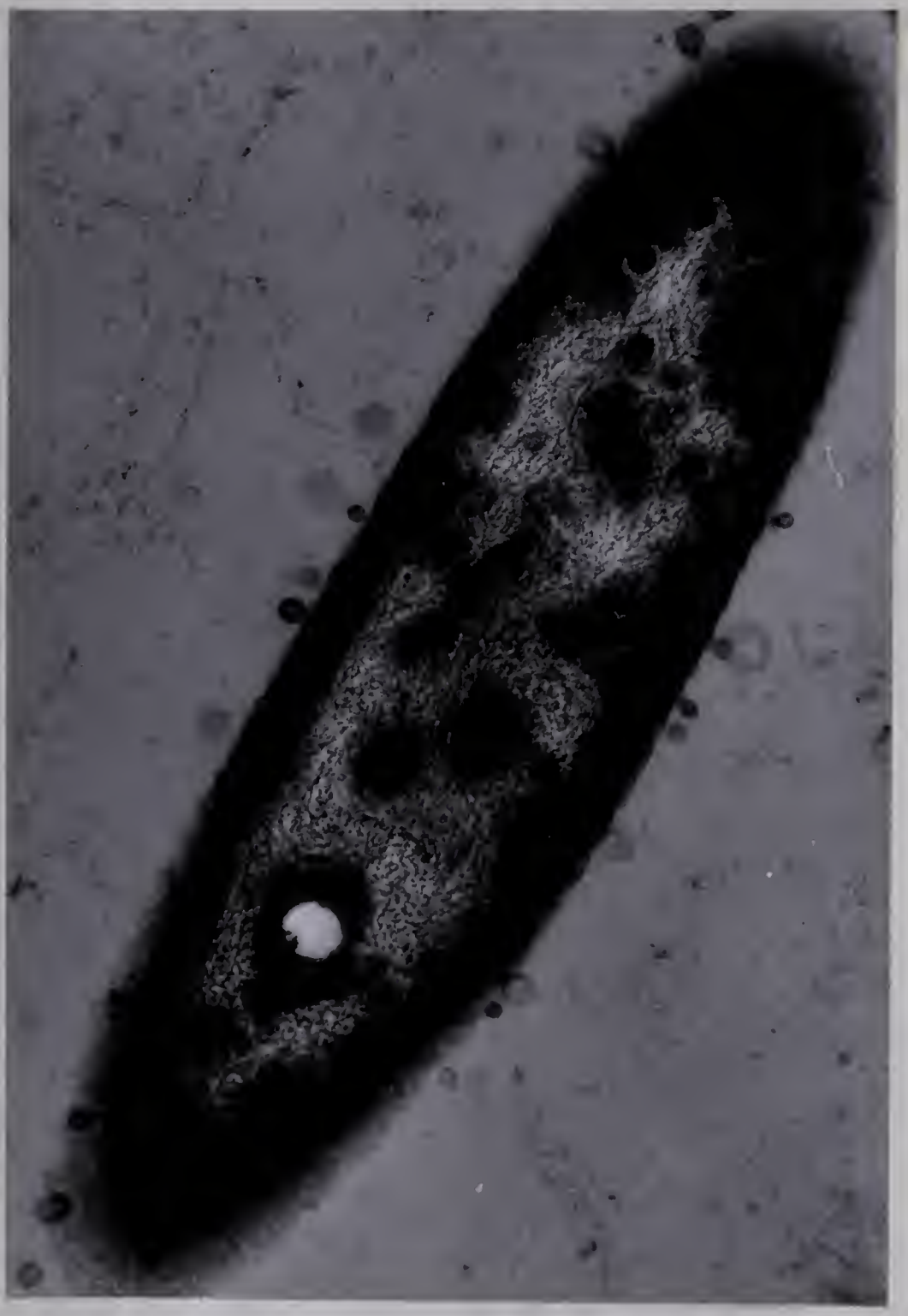

Figure 26. Electron Micrograph of MAC-264 Cells 105 Minutes after Mitomycin C Treatment.

For legend, see Fig. 22.

X 50,000.

Figure 27. Electron Micrograph of MAC-264 Cells 120 Minutes after Mitomycin C Treatment.

For legend, see Fig. 22.

X 80,000.

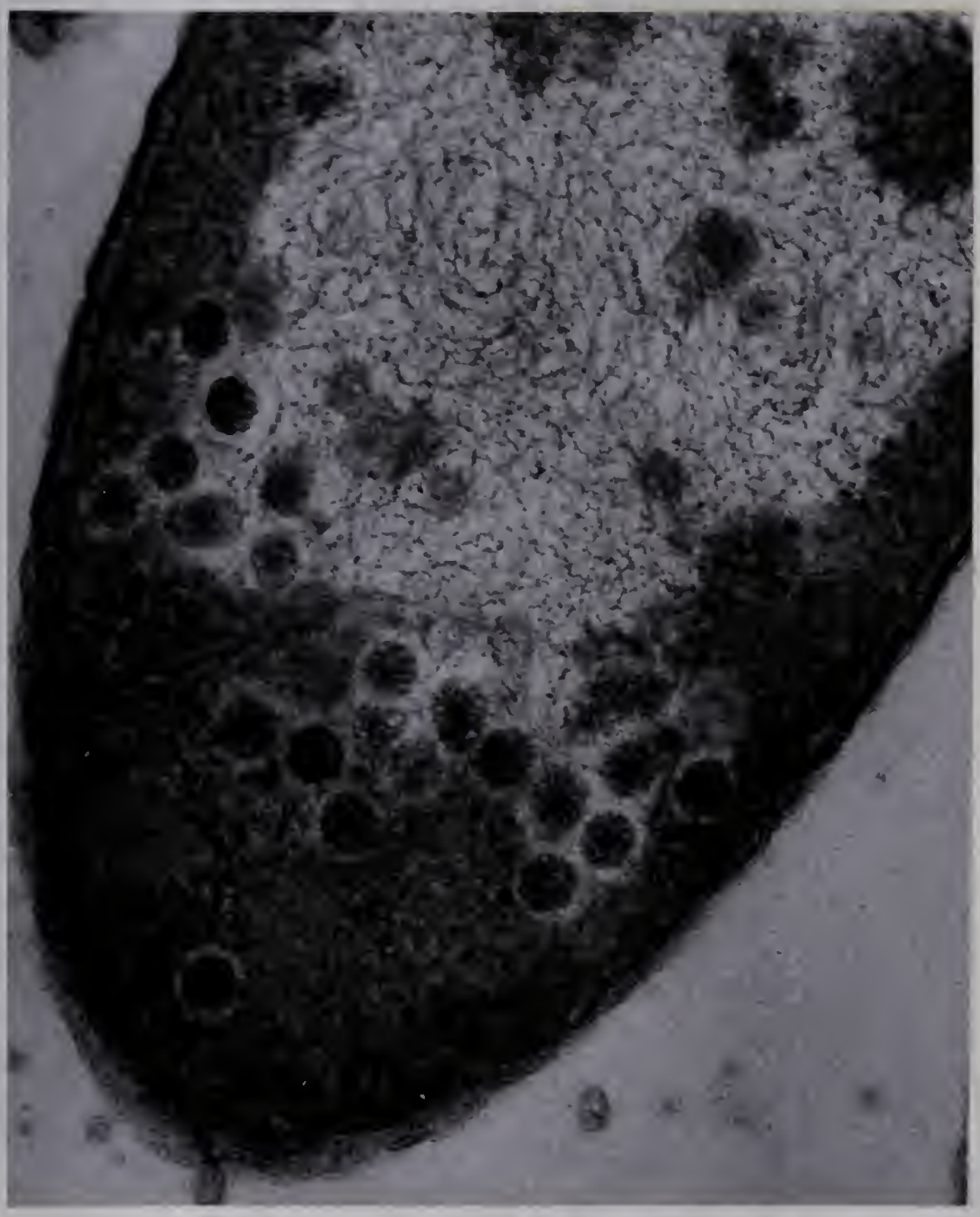

Figure 28. Electron Micrograph of MAC-264 Cells 150 Minutes after Mitomycin C Treatment.

For legend, see Fig. 22.

X 120,000.

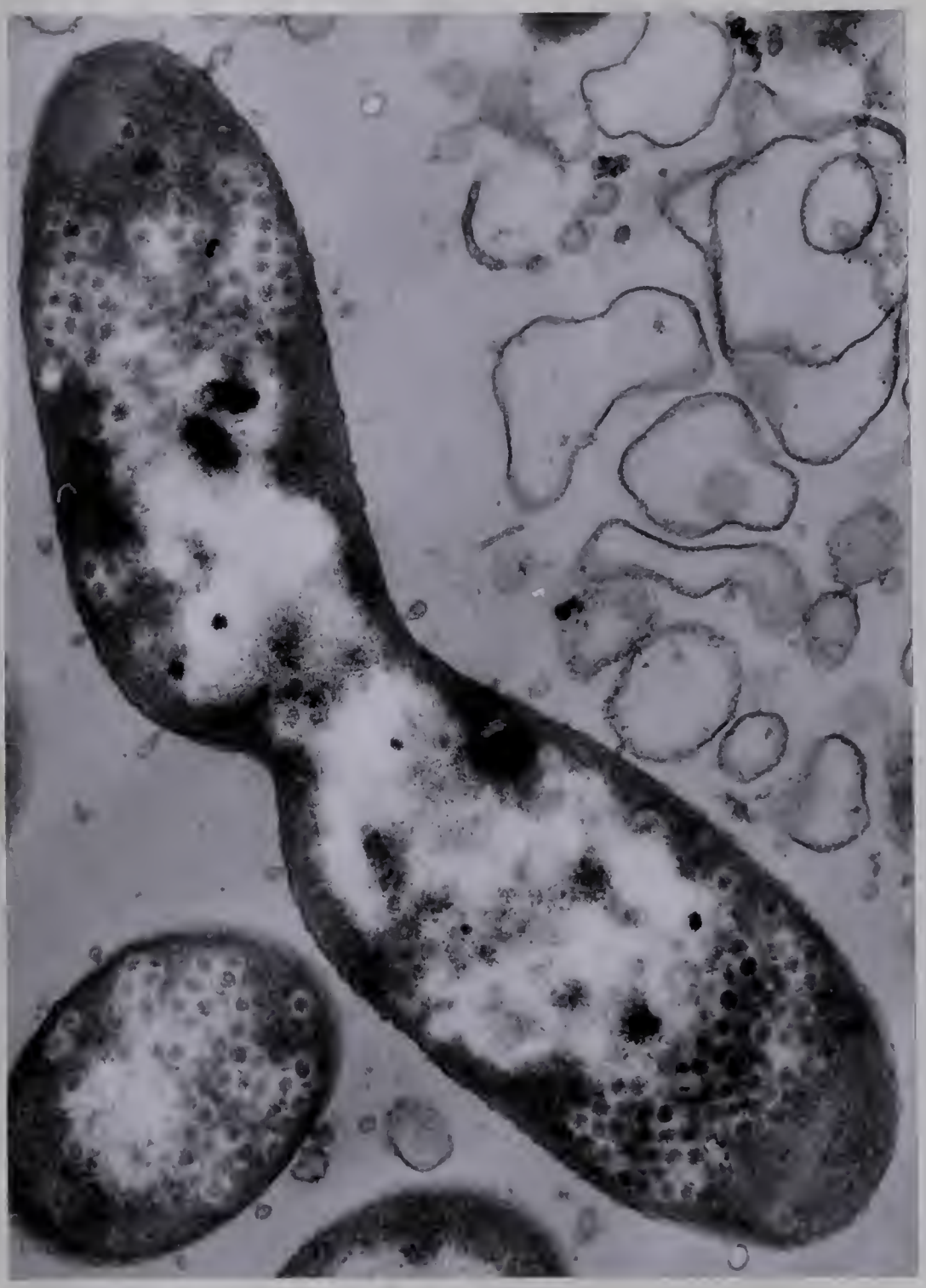

Figure 29. Electron Micrograph of MAC-264 Cells 150 Minutes after Mitomycin C Treatment.

For legend, see Fig. 22.

X 50,000.

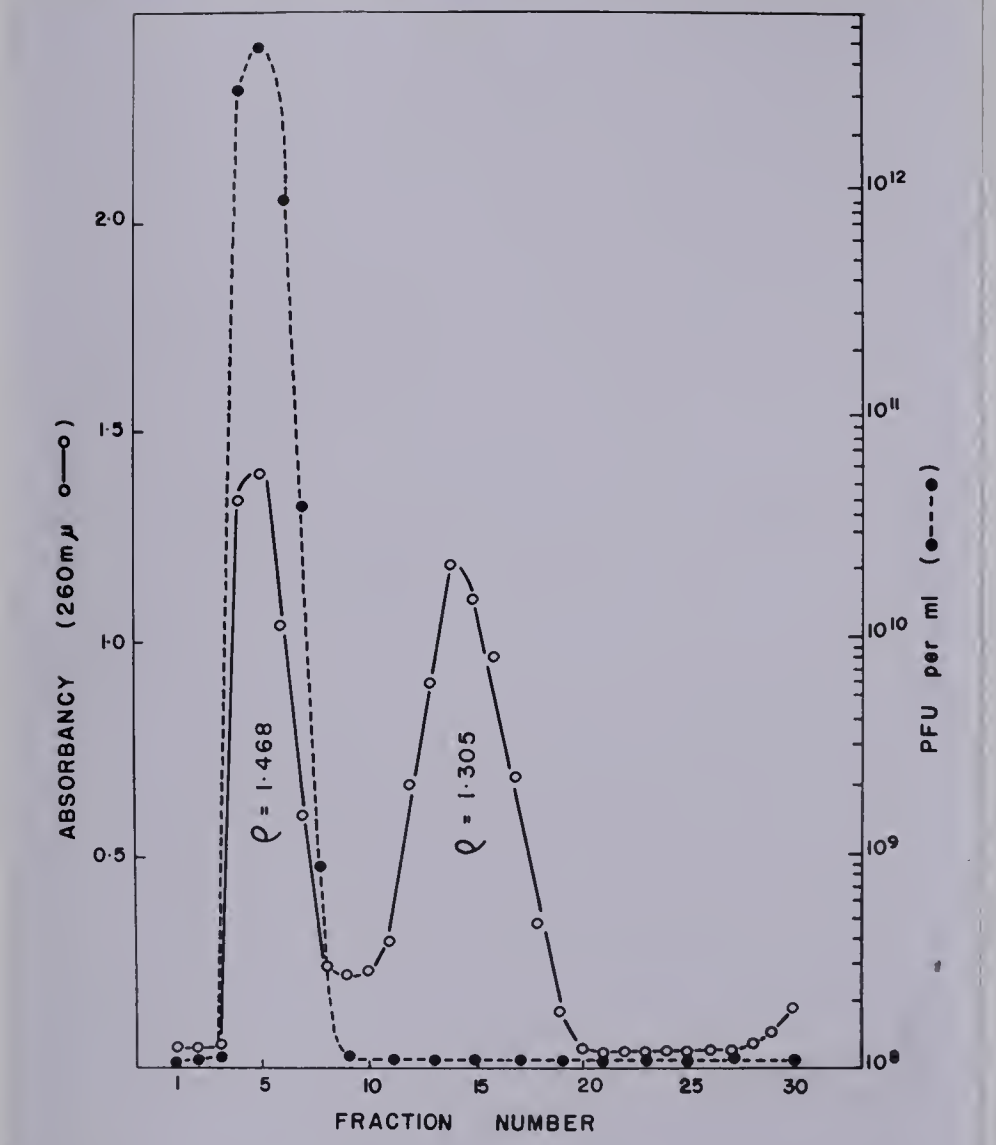
ABSORBANCY (260mμ ○—○)
PFU per ml (●---●)
2·0
1·5
1·0
0·5
10¹²
10¹¹
10¹⁰
10⁹
10⁸
ρ = 1·468
ρ = 1·305
1
5
10
15
20
25
30
FRACTION NUMBER

Figure 30. Analysis of MAC-264 Cell-lysates Collected 180 Minutes after MC-Treatment.

MC (2.5 μg/ml) was added to an exponentially growing MAC-264 culture in nutrient broth. Incubation continued in a 37° water-bath shaker for a further 180 minutes, when complete lysis of MAC-264 cells has already occurred. The bacterial lysates were collected by centrifugation and analysed by using CsCl equilibrium centrifugation technique.

Figure 31. Electron Micrograph of Phage ∅-MC.

MC (2.5 µg/ml) was added to an exponentially growing MAC-264 culture. One hundred and eighty minutes later, the cell-lysates were collected and analysed in a CsCl gradient. A band formed at ρ = 1.468 g/ml was dialysed and the micrograph taken. X 400,000.

was examined in an electron microscope, and found not to contain any phage Ø-MC, but consisted of cell debris and phage tail-like structures.

14. Electron Microscopic Examination of the Components in MC-treated MAC-264 Cell Lysates:

An electron micrograph of material banding at ρ = 1.468 g/ml is presented in Fig. 31. The nucleocapsid was of a regular hexagonal outline and measured about 55 mμ in diameter. There was a short wedge-shaped tail attached to one apex of the head. The morphology of this induced phage Ø-MC is similar to that of coliphage T_3 described by Bradley and Kay (1960) and T_7 reported by Davison and Freifelder (1962).

Another electron micrograph of material banding at ρ = 1.305 g/ml is presented in Figs. 32 and 33. Here some phage tail-like structures and cellular debris can be seen. These phage tail-like structures resemble pyocins described previously by Ishii et al. (1965) and Kageyama (1964).

15. Determination of the Chemical Compositions of Phages Ø-2 and Ø-MC:

Analysis of phages Ø-2 and Ø-MC nucleic acids proved to be DNA since phenol extraction gave a positive diphenylamine test and a negative orcinol test. The DNA content was further proved by their susceptibility to DNase, but not to RNase.

The DNA and protein content in these two phages were measured by diphenylamine reagent and Lowry's tests, respectively. From the standard curves of DNA and protein, shown in Figs. 34 and 35, the DNA and protein content were measured and the results are shown in Table III.

The average values of DNA, protein, particle weight and molecular weight per PFU of Ø-2 and Ø-MC were calculated and shown below:

Phage strain	DNA (μg)	Protein (μg)	Particle wt.	Molecular wt
Ø-2	1.4×10^{-10}	0.97×10^{-10}	2.37×10^{-10}	143×10^{6}
Ø-MC	0.77×10^{-10}	0.91×10^{-10}	1.68×10^{-10}	110×10^{6}

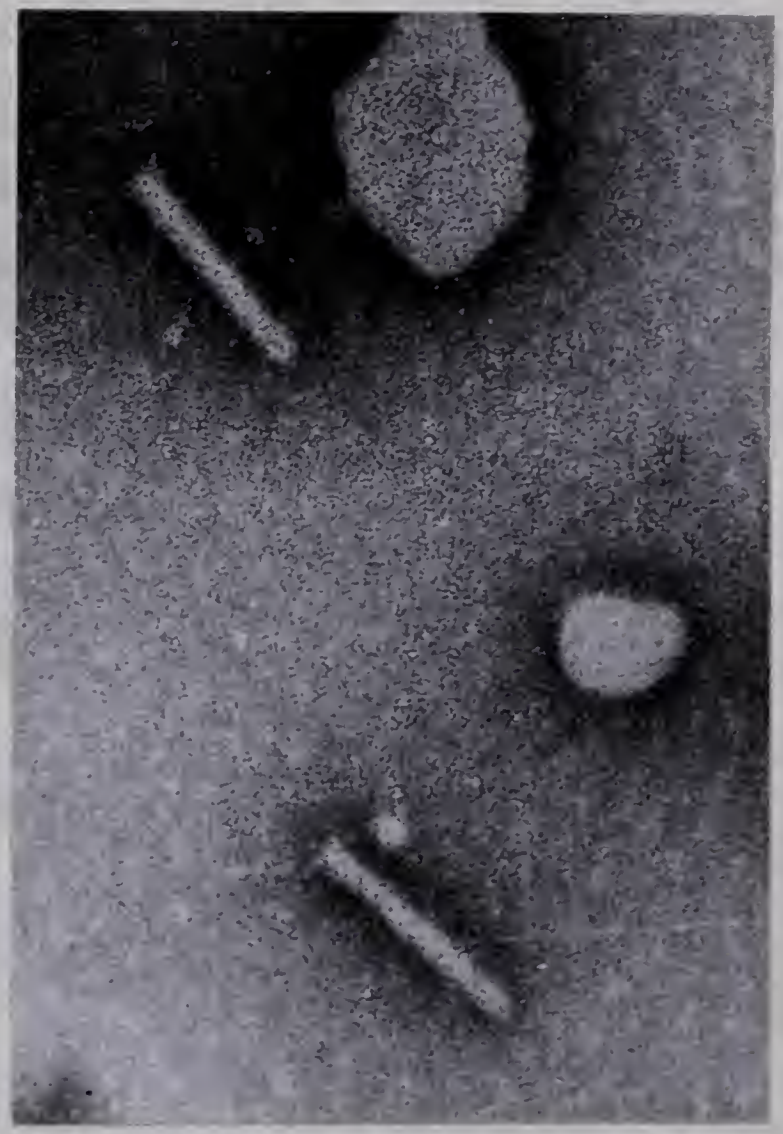

Figure 32. Electron Micrograph of Phage Tail-like Structures in MC-lysed MAC-264 Culture.

The preparation is similar to that in Fig. 31, except the material was banding at ρ = 1.305 g/ml.

X 200,000.

Figure 33. Electron Micrograph of Phage Tail-like Structures in MC-lysed MAC-264 Culture.

Same as Fig. 32.

X 200,000.

TABLE III

DNA and Protein Content in Phages ϕ-2 and ϕ-MC

Phage	PFU/5 ml X 10^{-11}	O.D. (600 mμ)	DNA (μg)	O.D. (500 mμ)	Protein (μg)
ϕ-2	5.5	0.20	78	0.19	54
	10.1	0.38	142	0.37	97
	12.0	0.45	170	0.45	128
	18.0	0.68	250	0.67	173
	30.0	1.16	424	1.10	292
ϕ-MC	5.5	0.08	30	0.13	35
	10	0.21	75	0.22	60
	16.5	0.38	120	0.38	100
	20	0.42	160	0.44	118
	23	0.44	165	0.54	140
	28	0.56	220	0.64	165
	31	0.58	228	0.75	195

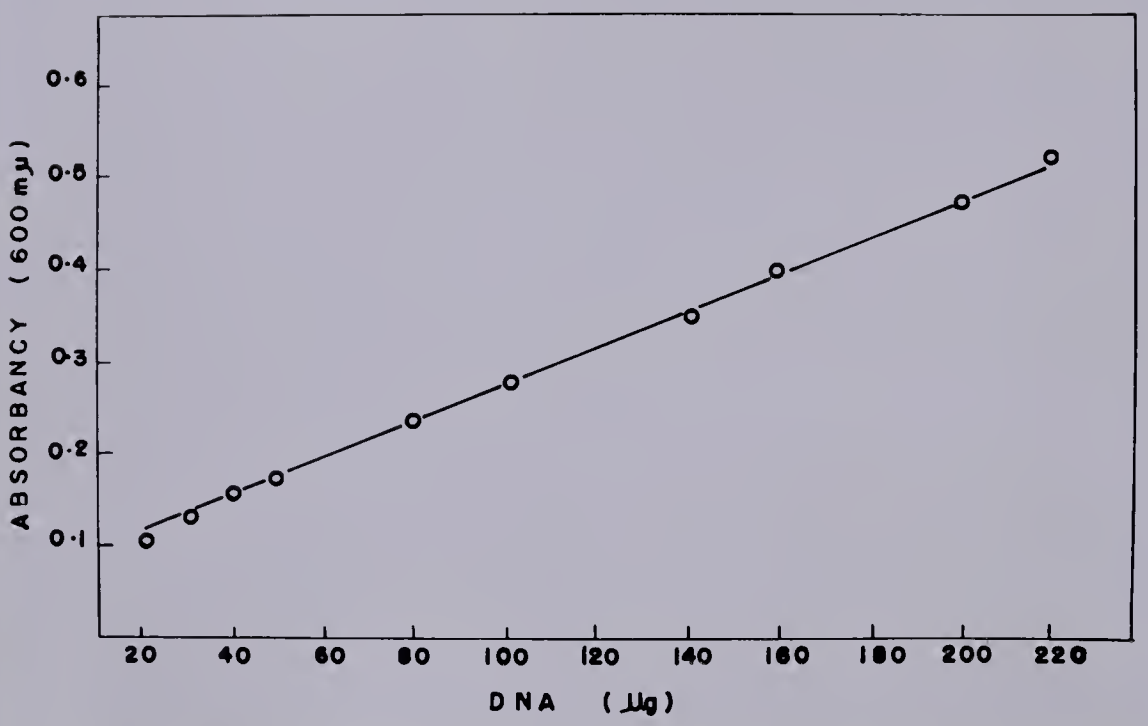
ABSORBANCY (600 mμ)
0·6
0·5
0·4
0·3
0·2
0·1
20
40
60
80
100
120
140
160
180
200
220
DNA (μg)

Figure 34. Standard Curve of DNA.

Calf thymus DNA was used to plot this standard curve.

One ml aliquots of solution of different concentration of DNA were added to 2 ml of diphenylamine reagent and heated at 100° for 10 minutes. Optical densities were measured at 600 mμ in a Beckman DB-G Grating Spectrophotometer.

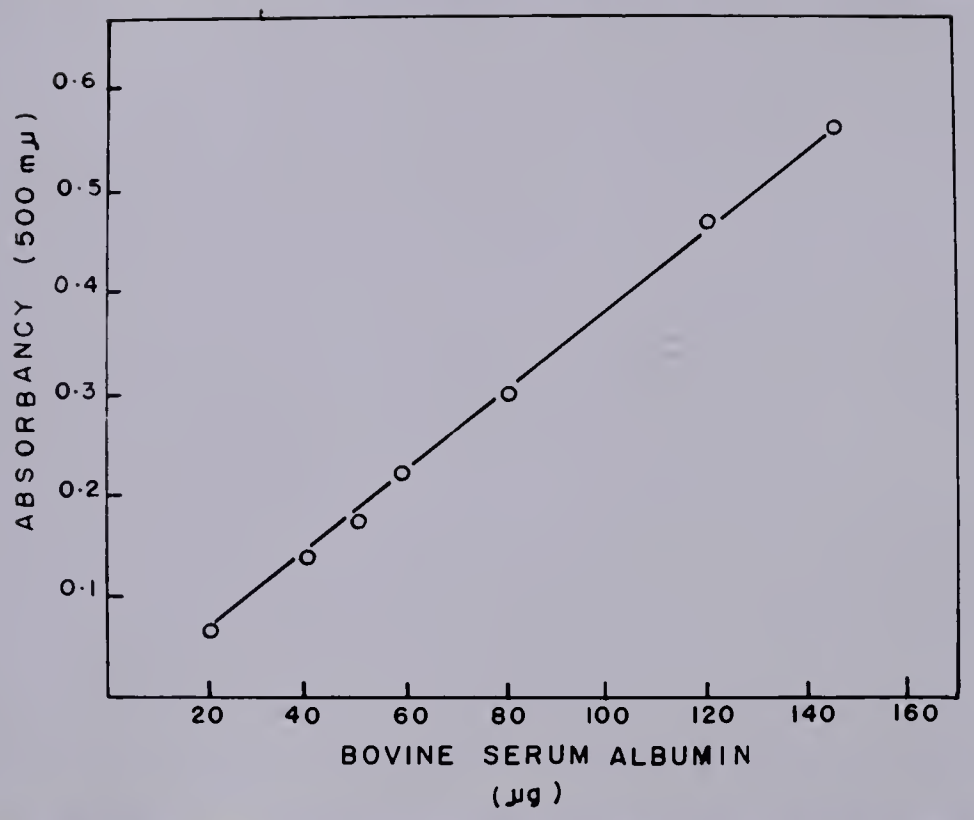
ABSORBANCY (500 mμ)
0·6
0·5
0·4
0·3
0·2
0·1
20
40
60
80
100
120
140
160
BOVINE SERUM ALBUMIN
(μg)

Figure 35. Standard Curve of Protein.

Bovine serum albumin and Lowry's Folin phenol reagent were used for plotting this standard curve.

The particle weights were calculated by assuming the efficiency of plating, which is defined as that proportion of viable phage particles which actually form plaques when plated, is near 100% (as reported by Hook et al, 1946, for T_2 phage). From their particle weights, the molecular weights could be calculated by multiplying the particle weights by Avagadro's number, i.e.,

$$\text{Mol. wt. of } \phi\text{-2} = 2.37 \times 10^{-16} \times 6.02 \times 10^{23}$$
$$= 143 \times 10^{6} \text{ daltons}$$

and,

$$\text{Mol. wt. of } \phi\text{-MC} = 1.68 \times 10^{-16} \times 6.02 \times 10^{23}$$
$$= 110 \times 10^{6} \text{ daltons}$$

The DNA-protein ratios of ϕ-2 and ϕ-MC were also calculated to be 59:41 and 45:55, respectively.

16. Comparison of DNA Densities of P. aeruginosa strain MAC-264 Cells and of Phages ϕ-2 and ϕ-MC

The DNA buoyant densities in CsCl of MAC-264 cells, phage ϕ-2, and phage ϕ-MC were found to be 1.728, 1.710 and 1.705 g/ml, respectively. The results are presented in Figs. 36, 37 and 38. A linear relationship between the buoyant density in CsCl of DNA and its mole percent guanine plus cytosine has been reported (Sueoka et al., 1959; Rolfe and Meselson, 1959). A curve was constructed by Schildkraut et al. (1962) by using more than 30 values obtained from the literature and of their own to show the linear relationship between density and G + C content in DNA. Using this curve and also the curve constructed by Boucher (1965), the mole percent guanine plus cytosine of the DNA's of MAC-264 cells, ϕ-2 and ϕ-MC were determined to be 68, 51 and 46 percent, respectively.

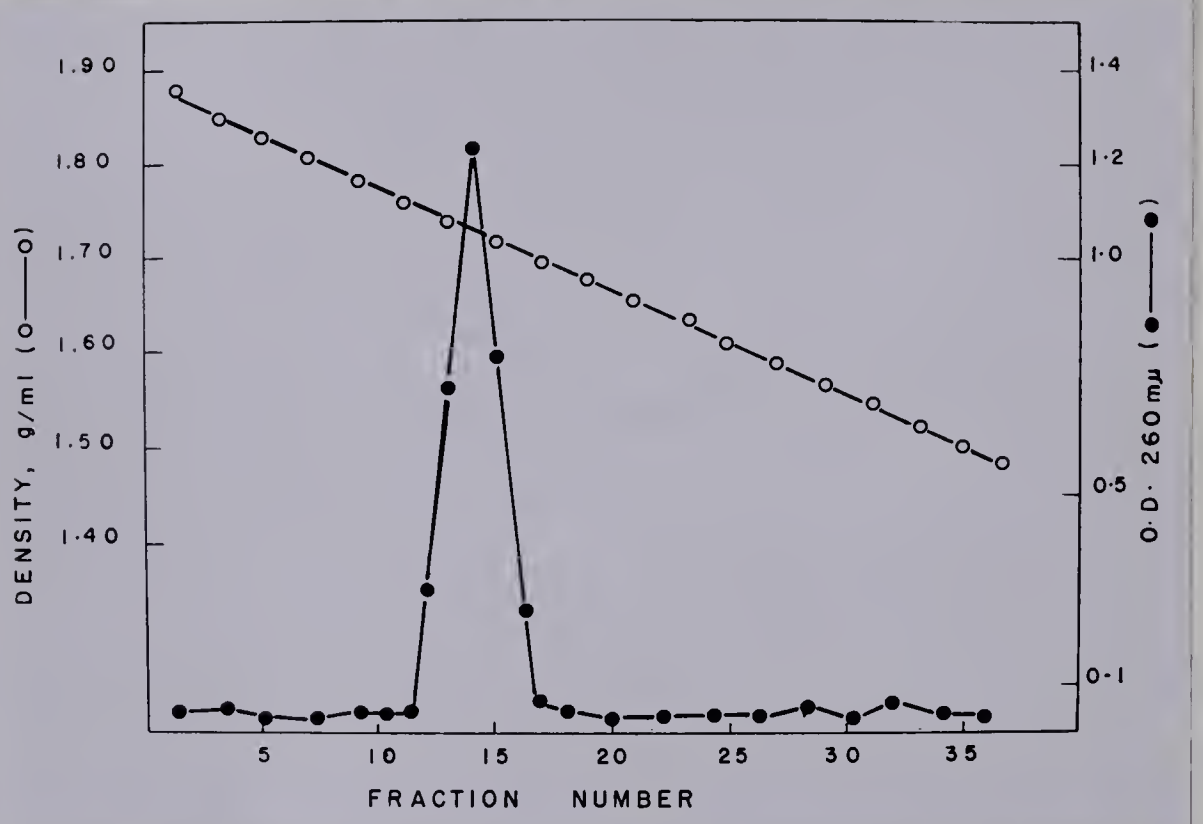
1.90
1.80
1.70
1.60
1.50
1.40
DENSITY, g/ml (o——o)
1·4
1·2
1·0
0·5
0·1
O·D· 260 mμ (●——●)
5
10
15
20
25
30
35
FRACTION NUMBER

Figure 36. Density Gradient Centrifugation of P. aeruginosa Strain MAC-264 Cell DNA.

The DNA was isolated from MAC-264 cells by Marmur's method.

The isolated DNA was centrifuged in a CsCl gradient at 35,000 r.p.m. for 3 hours. Fractions were collected by piercing the bottom of the centrifuge tube. Each fraction was determined for its buoyant density and optical density at 260 mμ.

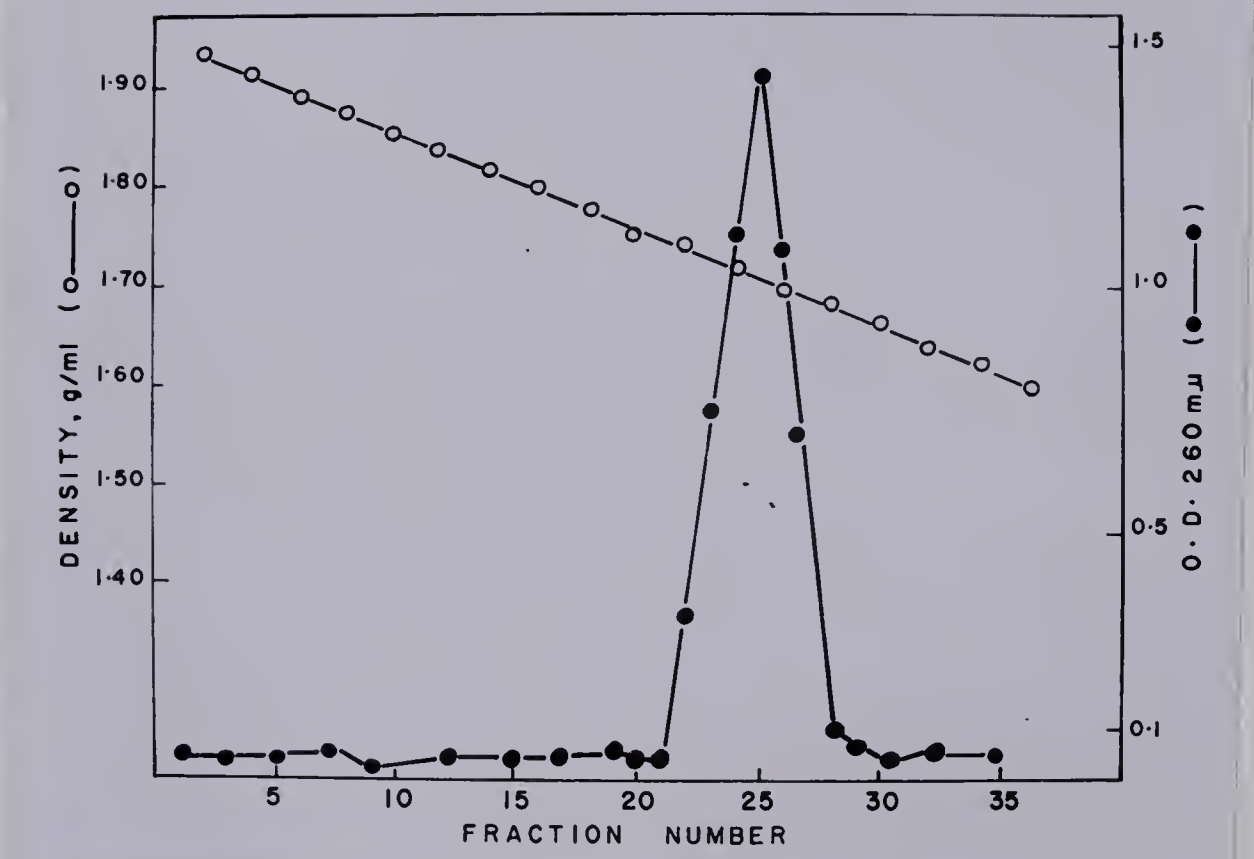
DENSITY, g/ml (o—o)
O.D. 260 mμ (●—●)
1·90
1·80
1·70
1·60
1·50
1·40
1·5
1·0
0·5
0·1
5
10
15
20
25
30
35
FRACTION NUMBER

Figure 37. Density Gradient Centrifugation of Phage ϕ-2 DNA.

The DNA was isolated from phage ϕ-2 by the phenol extraction procedure of Mandell and Hershey.

The isolated DNA was centrifuged in a CsCl gradient at 35,000 r.p.m. for 3 hours. Fractions were collected by piercing the bottom of the centrifuge tube. Each fraction was determined for its buoyant density and optical density at 260 mμ.

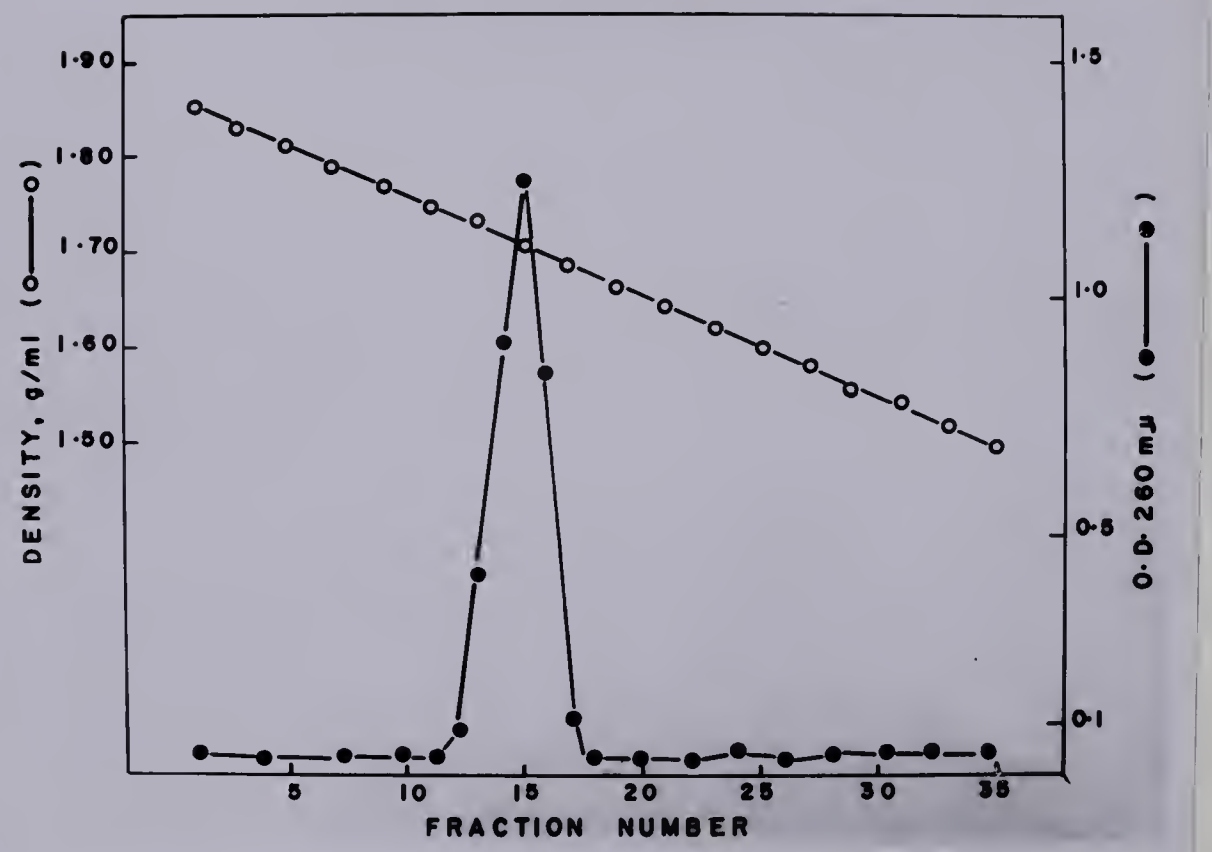
1·90
1·80
1·70
1·60
1·50
DENSITY, g/ml (o——o)
1·5
1·0
0·5
0·1
O·D· 260 mμ
5
10
15
20
25
30
35
FRACTION NUMBER

Figure 38. Density Gradient Centrifugation of Phage ϕ-MC DNA.

For legend, see Fig. 36.

These values agreed with that obtained by using the formula reported by Schildkraut, Marmur and Doty (1962),

$$\rho = 1.660 + 0.098\ (G + C\ \%).$$

DISCUSSION

The first step in the phage infectious cycle is its adsorption to susceptible bacterial cells, and therefore the specificity of the phage-bacterium relationship is, in most cases, determined by the adsorption process. In this study, the adsorption rate of phage ϕ-2 to Pseudomonas aeruginosa strain MAC-264 cells in the first 5 minutes follows the kinetics of a first-order reaction which agrees with the report of Krueger (1931) for coliphages. The adsorption rate is found to be 1.5 X 10^{-9} ml/minute which is very close to that of another P. aeruginosa phage 2 whose adsorption rate is about 1.55 X 10^{-9}ml/minute reported by Grogan and Johnson (1964).

However, adsorption of phages to susceptible bacteria is not equivalent to infection (Hershey et al., 1944; Puck et al., 1951), since the adsorption process involves at least two successive steps, the first of which is reversible. This may explain the phenomenon that only about one third of the killed cells become infective centers after antiserum treatment. The cells which do not form infective centers are thought to be killed by the attachment of the phage tails through the action of the enzyme, lysozyme, carried by the phage particles. (Weidel, 1951; Koch and Weidel, 1956).

The multiplicity of infection was chosen to be 6 to 9 in most of the experiments in this study. At such a multiplicity, according to Poisson's formula, less than 0.0005% of the bacteria are not infected, or, in other words, 99.9995% of the population are infected. This gives a uniform preparation for study.

In the T-even phage system of E. Coli, a decrease in turbidity is usually observed after phage infection (Doermann, 1948; Adams, 1959;

Stent, 1963). In contrast, the Pseudomonas phage system, the failure to cause clearing of the bacterial culture is not uncommon (Boucher, 1965). A possible explanation appears to be that lysis of the infected Pseudomonas cells, under the conditions given, is not synchronized, and also surviving cells continue to grow. The combination of these facts results in a lack of change in optical density. Or, another explanation may be due to abortive infection, i.e., infection accompanied by loss of the infecting phage particles and often death of the bacteria but not yielding any phage progeny (Benzer and Jacob, 1953; Gross, 1954).

Cohen (1948) one of the first workers to apply the methods of modern biochemistry to the problem of phage growth, first studied the nucleic acid metabolism of T_2-infected E. coli cells and found that net DNA synthesis in the culture comes to a halt immediately after infection, and that some 5 to 6 minutes later DNA synthesis resumes at a rate greater than that before infection. A similar result is obtained in this system, although the resumption does not begin until 10 minutes after infection. If one assumes all of this postinfection DNA synthesis concerns the manufacture of phage DNA, from the DNA standard curve (Fig. 33) and the DNA content in phage Ø-2 (1.4×10^{-16} g/PFU), about 50 phage DNA units are accumulated 30 minutes after infection when the intracellular phage particles appear. Comparing the T_2 system (Hershey et al., 1953) where 40 to 80 phage DNA units are present within the infected cells when the first infective progeny make their appearance, it can be seen that the phage pool rise is similar in these two systems. At the end of the rise period, a constant level of DNA is attained. This amount of DNA represents about 70 phage DNA units per infected cell. Since only one third of the infected cells are producing phage

progeny, 210 phage DNA units should be present in every cell which is producing phage. One-step growth curves (Figs. 7, 8 and 9) show that the burst size is about 120 to 140, therefore, there are about 70 to 90 phage DNA units not incorporated into infectious phage particles.

In contrast to the DNA synthesis of ϕ-2 infected MAC-264 cells, the synthesis of RNA comes to a halt 10 minutes after infection (Fig. 5). There is very little increase in the total amount of RNA during the remaining time when intracellular multiplication of the phage is actively under way. This stoppage in RNA synthesis reflects that there is a phage-induced termination of the formation of bacterial ribosomes (Cohen, 1948; Stent, 1963). Thus, whatever ribosomal particles are to be used for the processes of viral growth must, already be present in the cells before infection.

The protein synthesis in the ϕ-2 infected MAC-264 cells has a rate close to that of the uninfected cells for the first 60 minutes after infection (Fig. 6) and then slows down. Cohen and Anderson (1946) found that the overall protein synthesis in T_2-infected E. coli cells is by no means inhibited by phage infection and continues at its preinfection rate throughout the latent period and it is also known that a large amount of unidentified protein is synthesized in phage-infected bacteria immediately after infection (Adams, 1959, p. 263). The function of this protein is probably for synthesizing new phage progeny. This gives a reasonable explanation of the high level of protein accumulated in the infected cells. (Fig. 7).

The one-step growth curves of phage ϕ-2 in MAC-264 cells (Figs. 7, 8 and 9) show a latent period of 45 minutes, followed by a rise period of 30 to 35 minutes. Thus, the entire cycle is completed in

approximately 75 to 80 minutes. Comparing these values with other P. aeruginosa phages reported by Feary et al. (1964) and by Grogan and Johnson (1964), this phage has a slower rate of replication. But the burst sizes in all these cases are about the same, 120 to 150.

In the study of intracellular growth, one would expect to have a much lower initial value of infective centers after treatment with chloroform (Figs. 7, 8 and 9). The relatively high value for the initial level obtained in this study, indicates that phages are probably released from the adsorbed bacteria by a reversible adsorption process mentioned earlier.

Chloramphenicol is an antibiotic known to have the inhibitory effect on the growth of a wide variety of bacteria, rickettsia and certain large animal viruses (Wisseman et al., 1954; Brock, 1961). Its activity against bacteria is primarily bacteriostatic, and it affects the multiplication of bacteriophages only indirectly through modification of the host cells. Hahn et al. (1957) showed that after removal of chloramphenicol, cultures of E. coli B/r requires a recovery time of about 90 minutes. But in P. aeruginosa system a recovery period of 50 minutes is sufficient for both bacterial cells and for replication of phage progeny. That chloramphenicol concentrations lower than 80 μg/ml cannot completely inhibit phage production indicates that phage protein is still being synthesized within the infected cells. Tomizawa and Sunakawa (1956) reported that phage DNA still can be synthesized if chloramphenicol is added at a sufficient concentration 4 minutes after infection to a T_2-infected E. coli culture. This indicates that most of the early enzymes, necessary for phage DNA replication, are synthesized within 4 mintes after

infection. In the MAC-264-ϕ-2 system, phage DNA is not synthesized if chloramphenicol, at a concentration of 80 μg/ml, is added 5 minutes after infection. However, there is some DNA synthesized when chloramphenicol is added 10 minutes after infection (Fig. 12). These results indicate that the early enzymes for ϕ-2 DNA replication are synthesized within 10 minutes after infection. Phage progeny can be synthesized 50 minutes after the removal of chloramphenicol (Fig. 13), and this is in agreement with the recovery time of the uninfected cells. These results show that the phage DNA injected into the cell is, *per se*, not affected by chloramphenicol.

The bacteriocidal effect of mitomycin C (MC) was investigated thoroughly by Iyer and Szybalski (1963, 1964). They found that MC acts similar to antitumor alkylating agents. Both *in vitro* and *in vivo* tests indicate that the DNA of MC-treated cells is "cross-linked" by MC and does not replicate. On the other hand, RNA and protein synthesis are not affected under appropriate conditions (Shiba et al., 1958, 1959; Sekiguchi and Takagi, 1959, 1964b) in which they reported that in MC-treated *E. coli* K 12 (λ) cells, a rapid alteration (within 5 minutes) of the DNA results. This alteration makes the cellular DNA a much poorer primer for DNA polymerase, but does not affects its ability to prime for RNA polymerase. They also reported that the DNA polymerase present in MC-treated *E. coli* K 12 (λ) cells exhibits the same properties as the DNA polymerase present in normal (untreated) cells. This finding indicates that the failure of replicating DNA in MC-treated cells is due to the alteration of the DNA rather than to the enzyme.

Since virulent phage ϕ-2 progeny are formed (Fig. 16) and

phage Ø-MC are induced in MC-treated cells (Fig. 20), it is evident that phage DNA replication can occur in the presence of MC. Sekiguchi and Takagi (1960) suggested that infected cells have an alternative pathway of DNA formation which is absent from the normal cells. This assumption that virus infection opens a new pathway for DNA synthesis is also supported by the finding of Barner et al. (1954) who demonstrated that the thymine-requiring E. coli cells could synthesize thymine and phage T_2 DNA in the absence of thymine upon T_2 infection. Pricer and Weissbach (1964a, 1964b) showed that the DNA of MC-induced phage, not like the cellular DNA, is still a good primer for DNA polymerase in the presence of MC. Or, in other words, the phage DNA is not affected by MC as the cellular DNA. The reason still remains obscure. A suggestion is made here that the "binding" of MC to DNA requires a specific structure and length of DNA, which are present in bacterial DNA but absent in phage DNA.

The ability of inducing temperate phage in lysogenic bacteria by MC has been reported (Otsuji et al., 1959, Levine, 1961; Korn and Weissbach, 1962; Seaman et al., 1964; Sutton and Quadling, 1963; Endo et al., 1965; Welker and Campbell, 1965). Many other chemical and physical agents are also shown to have this ability (Lwoff, 1953). All of these agents appear to have a characteristic in common, i.e. the interruption of host DNA synthesis. Since the DNA of the temperate phage is attached to the host DNA (Jacob and Wollman, 1959; Calef and Licciardello, 1960; Campbell, 1958; Cowie and McCarthy, 1963), it appears that any agent able to exsect the host DNA is also likely to release the temperate phage DNA and probably results in phage replication.

The induction of Bacillus stearothermophilus cells (Welker and Campbell, 1965) has a very narrow range and low concentration of MC (0.05 to 0.1 μg/ml). But in most other systems (Pricer and Weissbach, 1964a, 1964b; Seaman et al., 1964) and in this study, a higher concentration (1 to 3 μg/ml) is required. These systems also have a much wider range of concentrations of MC for induction (1 to 10 μg/ml).

The rise in turbidity of the culture, during the first 90 to 105 minutes after addition of MC, indicates that protein synthesis does continue during the process of induction. This point is also supported by the protein determination. Therefore, one would expect to have an increase in size of the bacterial cells after MC-treatment and this is found to be the case in both light and electron microscopic observations. However, the cells never elongate to a level up to 300 μ, as reported by Reich et al. (1961) and by Suzuki et al. (1967) in E. coli 15 T and in E. coli B cells. The reason is understandable, since both E. coli cultures used by them were non-inducible whilst P. aeruginosa strain MAC-264 cells are lysogenic and produce phage particles upon treatment with MC. Part of the protein synthesized in the induced cell will be phage protein. Figure 19 shows that in MC induced cells, protein synthesis proceeds at a slightly lower rate than in the control, and it stops at the time of lysis.

From the DNA increase in the MC-treated cells (Fig. 18) and from the DNA standard curve (Fig. 33), one can measure an increase of about 40 μg DNA in 120 minutes. The DNA content of a Ø-MC particle is calculated to be 0.77×10^{-10} μg. Assuming that all the DNA synthesized in the presence of MC are phage Ø-MC DNA, 52×10^{10} phage DNA units are

accumulated in 10 ml of bacterial culture which contains about 1.5 X 10^9 cells. If all the cells are induced, there are about 340 phage ϕ-MC particles produced in every induced cell. This value is higher than that found in electron microscope pictures (Plates 8 and 9). This difference is explainable since some of the DNA remains in the DNA pool, and not all the DNA synthesized is incorporated into complete phage.

The buoyant density of phage ϕ-MC in CsCl is found to be 1.468 g/ml which agrees with that of another Pseudomonas phage gh-1 (ρ = 1.470) with a similar structure to ϕ-MC reported by Niblack et al. (1966) and by Lee and Boezi (1966). The buoyant density of phage ϕ-MC DNA in CsCl is found to be 1.705 g/ml which is the same as another Pseudomonas phage ϕ-16, isolated from sewage by Boucher (1965). However, phage ϕ-16 forms plaques on MAC-264 while phage ϕ-MC does not. Thus, they are not identical, although, they have the same DNA density and similar morphology (Yamamoto, 1967).

The linear relation between density and G + C content in DNA was independently established by Sueoka et al. (1959) and Rolfe and Meselson (1959), and further extended by Schildkraut et al. (1962) by determination of the densities in CsCl and base compositions of more than 50 strains of different organisms. Using this method, the G + C content in P. aeruginosa strain MAC-264 cells was found to be about 68% in this study, and this value agrees with that found by Schildkraut et al. (1962) and Mandel (1966). Both chemical analysis and density calculation were used and they found no unusual bases. Assuming the temperate phage, ϕ-MC, does not contain any unusual base, its G + C content is calculated to be about 45.5% which is much lower than that

of the host cell (68%). If it contains any unusual base, the G + C content will be even lower, since all the unusual bases found have higher density than that of the usual ones. Therefore, this is another example of a temperate phage that has a DNA composition different from that of its host. Other examples were shown by Marmur and Cordes (1963) in phage PBS2 in Bacillus subtilis, and by Welker and Campbell (1965) in a temperate phage induced by mitomycin C in Bacillus stearothermophilus.

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